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CONTENTS.

PATHOLOGY AND BACTERIOLOGY.

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THE THERAPEUTIC USE OF TRYPARSAMID IN NEUROSYPHILIS.*

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In the latter part of 1919, we entered on a study of the treatment of syphilis of the central nervous system with a view to the development and use of new drugs. At the suggestion of Dr. Wade H. Brown, we began our study with tryparsamid, the sodium salt of *N*-Phenylglycineamid-*p*-arsonic acid, $C_6H_4(NHCH_2CONH_2)\cdot(AsO\cdot OH\cdot ONa)$, which was first made by Jacobs and Heidelberger[1] in 1915. The biologic action of this substance has been studied experimentally by Brown and Pearce[2] in normal animals and in animals infected with trypanosomes and with the spirochetes of relapsing fever and of syphilis. Tryparsamid had also been used in a comparatively small group of patients for the treatment of syphilis other than that of the central nervous system, first by Louise Pearce and later by Keidel and Moore; and at the time our investigation began, it was about to be tested by Dr. Pearce in the treatment of human trypanosomiasis.[3]

From the work which had been done, it was known that single doses of the drug as large as 5 gm. could be administered with safety except for the occasional occurrence of transient symptoms of amblyopia.

* Tryparsamid is described under New and Nonofficial Remedies in this issue. The substance is not yet offered for sale.

* The expenses of this research were partly defrayed by grants from the U. S. Interdepartmental Social Hygiene Board. The authors have had the valuable assistance in this research of Drs. M. F. Kasak, R. L. Kenney and A. K. Viner.

* The work reported in this paper is a part of an extended investigation of the therapy of neurosyphilis by Drs. Loevenhart and Lorenz, begun in 1919 at the University of Wisconsin. The drug used for the work was supplied by The Rockefeller Institute for Medical Research.

1. Jacobs, W. A., and Heidelberger, M.: J. Am. Chem. Soc. **41**: 1581, 1917; J. Exper. Med. **30**: 411 (Nov.) 1919.

2. Brown, W. H., and Pearce, Louise: J. Exper. Med. **30**: 417, 437, 455, 483 (Nov.) 1919; **33**: 193, 1921.

3. Pearce, Louise: J. Exper. Med. (supplement) **34**: 1 (Dec.) 1921.

It had also been found that old or indolent lesions of syphilis disappeared promptly under treatment with tryparsamid, that patients showed a marked improvement in physical condition, and that a positive blood Wassermann reaction could be reduced to negative. Early primary and secondary lesions, on the other hand, responded less favorably, so that the employment of the drug in this class of patients was discontinued.[4]

The introduction of tryparsamid into the therapy of syphilis was not based primarily on its spirocheticidal action, which was comparatively feeble, but on certain unusual features of toxicologic and therapeutic action observed in experimental animals, such as the promptness of recovery from toxic injury, tolerance to repeated doses, a marked tonic effect, and the ability of the drug to induce resolution and healing of syphilitic lesions, even in the presence of actively motile spirochetes, but without increasing the liability to the occurrence of a generalized disease.

There were additional facts to recommend the use of this drug in the treatment of syphilis of the central nervous system. From the results obtained by the administration of toxic doses of tryparsamid to laboratory animals there was abundant evidence to show that the drug possessed an affinity for the tissues of the central nervous system, and it appeared that, with proper regulation of dosage, the difficulties of penetration hitherto experienced with arsenicals and other drugs might be overcome and that the affinity of the drug for these tissues might be utilized for therapeutic purposes. This had been accomplished in rabbits with trypanosomiasis, in which there is a distribution of organisms and of lesions in the central nervous system comparable to those of cerebral syphilis in man.

Statistics show that approximately 5 per cent. of persons with syphilis later develop paresis. It is now well known that from 30 to 40 per cent. of all syphilitic patients show positive spinal fluid findings at some time in the course of the disease; yet, from clinical experience, we know that the incidence of paresis is far below 40 per cent. Therefore, many of these cases must be arrested at some time in the course of the disease and paresis spontaneously prevented. In such instances,

4. Personal communication to the authors.

it seems permissible to hypothecate a defensive mechanism of some kind which protects nervous tissues, a reaction which either makes the cell more resistant or the toxins less destructive: in either event, a protective agency that is endogenous. Such speculation allows for the conception of remedies that might not be directly inimical to the infecting organism, but act as stimulants to such a natural defensive mechanism.

Moore[5] has recently pointed out that the relative infrequency of clinical neurosyphilis in women, as compared with men, may be due to the incidence of pregnancy. It has long been known that pregnancy modifies the reaction to syphilitic infection. It may be that physiologic processes incident to pregnancy tend to prevent the disease.

Dunlap[6] states that the treatment of general paresis may not be absolutely hopeless; that some of the spirochetes at least are probably accessible, and that we may need a modified therapeutic agent different from that which succeeds with the ordinary syphilitic strains.

The tryparsamid used in our work has been furnished us by The Rockefeller Institute; and Drs. Brown and Pearce have placed at our disposal all of their experience with the compound.

The drug is a white, crystalline solid, extremely soluble in water. It is odorless and tasteless and possesses practically no local irritating action. On intravenous injection of a 50 per cent. solution in the anesthetized animal, no observable changes are noted in the blood pressure or respiration, and the drug is remarkably inert as to immediate effects.

We have confirmed many of the laboratory findings of Brown and Pearce and have studied the rate of excretion of tryparsamid in man and animals. We have made a study of the effect of tryparsamid on the retina in animals and have studied the clinical use of the drug in neurosyphilis and other forms of late syphilis, but the present report deals in the main with cases in which there was involvement of the central nervous system. The laboratory findings and the details of

5. Moore, J. E.: Studies in Asymptomatic Neurosyphilis: Apparent Influence of Pregnancy on Incidence of Neurosyphilis in Women, Arch. Int. Med. **30:** 548 (Nov.) 1922.

6. Dunlap, C. B.: Spirochetes in General Paralysis, Arch. Neurol. & Psychiat. **8:** 589 (Dec.) 1922.

the clinical work will be published elsewhere, and we desire to present herewith merely a statement of the therapeutic results which we have thus far obtained with the drug.

Type of Cases.

A list of the type of cases treated is shown in Tables I and II. Table I shows patients committed as insane, and Table II shows the noncommitted ambulatory patients. Most of our patients were insane and the cases were diagnosed by us as paresis. In view of the rather generally held conception of paresis, especially from the standpoint of the futility of treatment, it seems desirable for us briefly to define this disease. It is our clinical experience, largely borne out by histologic studies, that the demarcation between meningovascular syphilis and paresis is not very clear. Excepting the extremes of these conditions, the transitional point, or mean, is a matter of opinion. We, therefore, have fixed a syndrome which we regard as paresis, preferring to discard entirely this term because its original use was determined in a great measure by a psychotic manifestation. In our work we include under paresis any case of syphilis of the central nervous system that has a certain physical symptom-complex and which is associated with definite mental symptoms of sufficient severity to warrant the conclusion that a psychosis exists. This psychosis may be varied in its symptoms; most common is a general dilapidation shown by a lessened interest and capability, with a proneness to confusion and faulty memory for recent events, associated with an emotional instability bordering on a childlike happiness or simplicity. These placid mental states are frequently interrupted by sharp periods of excitement. In some cases a manic-like picture is seen, but most common and characteristic is a feeling of happiness or well-being. This may be actually expressed or evidenced by conduct and may be inferred from the topic of conversation selected by the patient or his general manner of speech. The carelessness and indifference of the patient relative to his immediate circumstances and the future are also characteristic mental symptoms of paresis. The classical picture of megalomania or delusions of great wealth, great physical strength or great attainment are relatively uncommon today. This is probably due to our recognizing paresis without these

expansive ideas that were essential for the picture as recognized years ago. To sum up, in the mental symptoms associated with the physical signs of tremors, disturbed deep reflexes, pupillary anomalies, speech defects, etc., that complete the picture of paresis, most stress would be laid on definite changes in character or personality; mental or intellectual indolence; mild or marked euphoria; memory disturbances; confusion; excitement; delusions; hallucinations, etc. In addition to this syndrome, a completely positive serology in both blood and spinal fluid in an untreated case adds the other elements essential for our diagnosis of paresis.

Next in order to the long-standing institutional cases we have included in our series twelve fairly early paretics—patients that were committed because of some serious conduct disorder, regarded and easily recognized as insane by laymen. These cases differed from the first mentioned in that the history of mental disorder rarely extended further back than six months previous to admission. This group comprised, largely, younger adults, and were mostly ex-service men. Their psychotic manifestations were usually quite acute, and in some instances presented a picture of manic excitement. The physical signs, especially tremors and loss of weight, were pronounced. Hyperreflexia was also especially marked. Some of these were cases that in the old days were referred to as fulminating paresis because of great excitement, rapid loss of weight, and death from exhaustion in a matter of one or two months after admission of the patient to a hospital. Quite frequently these cases are mistaken for manic excitement. In this group the pupillary anomalies were but slightly in evidence. In some cases the changes approached the Argyll Robertson type, but rarely was this sign bilaterally present. Usually one found a relative slowness and limited excursion in response to light as compared to the response in accommodation, a condition which might be termed Argyll Robertson-like. In some cases, no pupillary anomalies were found, although most cases manifested some changes, such as irregularity in outline or an inequality or loss of sympathetic dilatation. Tremors were very constant and especially noticeable about the lips and tongue. These were usually made more evident by emotional upset or excitement. Speech incoordination was also very frequent, and, again intensified by strain and effort at conversation. Among these cases were some that might be regarded as classical from the

standpoint of expansiveness. Most of the patients bordered on a euphoric condition. A few were inclined to be depressed or emotionally unstable. In every case the physical and mental symptoms, together with completely positive serologic findings, gave sufficient evidence clearly to warrant the diagnosis of paresis as seen at any hospital for the insane.

It has been a common experience since serologic methods have been widely used to find patients giving all the laboratory findings of paresis without any suggestion of mental disturbances. For such cases we have reserved the term of asymptomatic paresis. Of these, we had fourteen that were entirely free from mental symptoms. Usually these patients sought a physician or a hospital because of a "nervous breakdown." Most of them, knowing of their syphilitic infection, had a well-defined fear that it was active. Among these were also patients that had been treated extensively but were serologically positive when referred to us. In these cases, the physical findings were relatively few or poorly defined. Those usually found were quite active deep reflexes and slight tremors. Headaches and fatigability are frequently early complaints in the history. The general complaints are of such a character that these cases might easily be passed by with the diagnosis of neurasthenia or psychoneurosis. In such cases, the diagnosis is definite only when the serologic findings are completely positive.

We had in our series five cases of frank tabes dorsalis and five cases of taboparesis.

We designate another group, under the general heading of syphilis of the central nervous system, as meningovascular syphilis. In this group we include all the cases that are generally referred to as cerebrospinal syphilis. In these the clinical syndrome is headache, ocular palsies, ptosis, dizziness, epileptiform seizures, transient or permanent cerebral focalization, such as spastic paraplegia of the upper or lower extremities, unilateral deafness, paralysis, and atrophy of the tongue with speech disturbances and impairment of deglutition. Insomnia, irritability, loss of ambition and an inability to concentrate are also quite common with a cerebrospinal fluid in which the serologic findings are quite different from those so commonly seen in paresis. In this group the cell count may be normal or greatly above that encountered in paresis. The Wassermann reaction of the spinal fluid is rarely posi-

tive in 0.1 c.c., usually requiring from 0.5 to 1 c.c. for a positive result. The colloidal gold test is also fairly characteristic in showing a maximum response in the fourth, fifth and sixth tubes. Of the meningovascular type, we had ten patients.

In addition, we had in our series nine cases that we have designated as late syphilis without central nervous system involvement. These

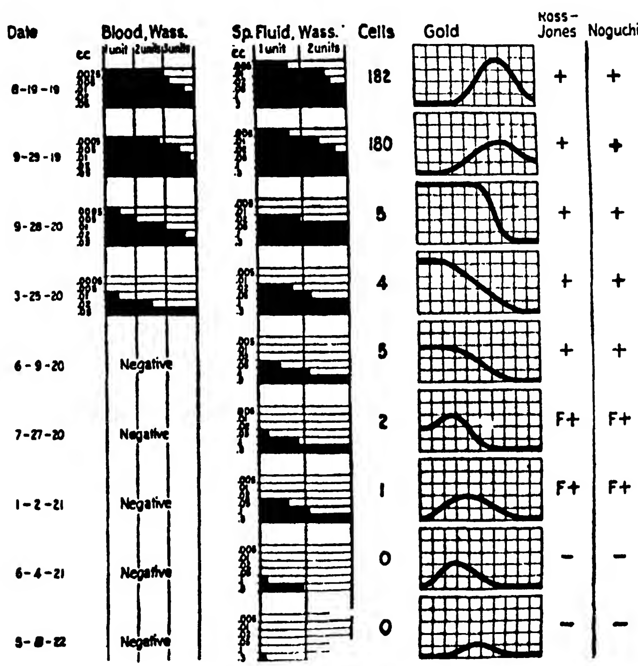

CHART 1. Observations in a case of manic excitement, later diagnosed general paralysis, committed to the State Hospital for the Insane. Over a period of two years, the patient was given three courses of tryparsamid and five of mercuric salicylate. After nine months of hospitalization, the patient was discharged, as the case was clinically arrested, and has continued to earn a comfortable living, without return of symptoms. The serologic findings, as seen in the last puncture, were completely negative. At the beginning of treatment the patient weighed 142 pounds (64 kg.), and at the end, 176 pounds (78 kg.). In this chart and the accompanying charts, black indicates the degree of Wassermann positivity. The length of the black lines shows the number of units absorbed. All Wassermann tests were made with varying amounts of serum or of cerebrospinal fluid, as indicated. Plus signs mean a positive globulin test. F + indicates a faintly positive reaction; —, a negative globulin test. Numerals in the "Cell" column indicate the number of lymphocytes per cubic millimeter.

comprise systemic syphilis, general in its character, such as congenital syphilis, cardiovascular syphilis, tertiary skin lesions and gummas. Included herewith were four cases of generalized syphilis in which there had been severe reactions to neo-arsphenamin; one an almost fatal case of dermatitis exfoliativa; another one of severe jaundice.

Methods.

The work began with a study of the action of tryparsamid in late paresis. It was obvious that we could not expect any great clinical improvement in the late cases. In these, the full measure of brain damage in the form of extensive parenchymatous degeneration had been done. Therefore, it was clear that our sole criteria of improvement would be the laboratory findings, which, for this reason, assumed great importance. In the earlier cases, therapeutic efficiency was estimated from the definite demonstrable clinical improvement and the serologic changes. There was first obtained the constant or basal serologic level by a series of examinations of both the blood and the spinal fluid before treatment started. Most of our patients had received treatment at some time prior to our efforts, but in no case had any antisyphilitic treatment been given for six months previous to our initial examinations.

The blood Wassermann test is performed in a special way. We use a serial dilution of blood serum from 2.5 to 50 per cent. Of each dilution, 0.1 c.c. is used in the test. In addition to these varying amounts of serum, we use separately in each test with each dilution one and two units of complement. Preferring unheated serum, we therefore first measure the amount of native complement present in each dilution, and furthermore we measure any anticomplementary reaction that may be present in each of the various dilutions of serum used. The total set-up for one test of a serum comprises twenty test tubes. The final result gives us the minimum amount of serum that will fix both one, two or more units of available complement. In other words we are able to measure the amount of complement deviated or fixed and the least amount of serum required to fix such measured amount of complement. The other components of the Wassermann test, as made by us, are acetone insoluble, alcoholic ethereal extract of heart muscle as antigen; freshly obtained sheep cells in 1 per cent. suspen-

sion, and antisheep hemolysin produced in rabbits. On the day of the test, units are established by careful titration in which the time element employed in the final test is followed. The antigen used must conform to certain fixed requirements. It must not be anticomplementary or hemolytic in 0.5 c.c. of a 20 per cent. emulsion. Its fixing property is such that 0.1 c.c. of a 2.5 per cent. emulsion completely fixes two units of complement in the presence of a 0.03 c.c. pooled

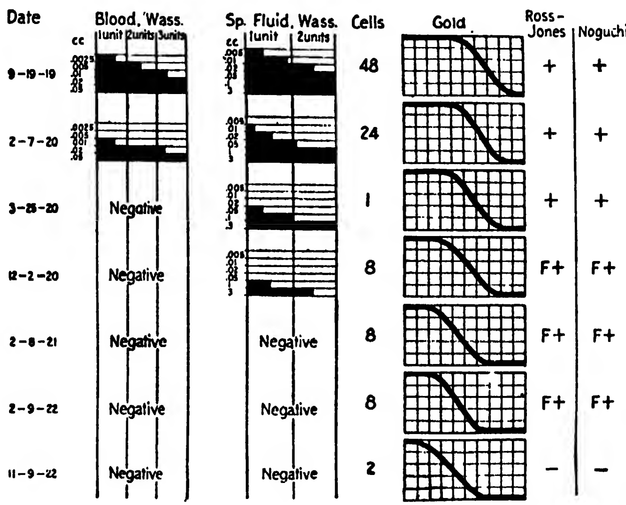

CHART 2. Observations in a case of late general paralysis committed to the State Hospital for the Insane. Treatment was begun in February, 1920. Only two courses of the drug were given; no treatment was given subsequent to December, 1920. The patient has returned to normal activity and has continued to earn a livelihood without recurrence of the clinical symptoms. The last puncture shows negativity of blood and spinal fluid, cells and globulin, but a persistence of the colloidal gold curve. At the beginning of treatment the patient weighed 165 pounds (75 kg.), and at the end 178 pounds (80 kg.).

positive serum. The dose used in the test is 0.1 c.c. of a 10 per cent. emulsion, or approximately ten units. Incubation at 39°C. is used throughout.

Our spinal fluid examinations consist of a Wassermann titration similar to that already described for blood serum. In addition, a cell count is made on the freshly drawn fluid, and a globulin estimation is made, the Noguchi butyric acid test and the contact test of Ross-Jones

being employed. The colloidal gold test is done in accordance with the generally accepted method; that is, serial dilutions of spinal fluid, beginning with a 1:10, then 1:20, 1:40, 1:80, etc., in all, ten tubes being used. To each of such dilutions there is added 5 c.c. of the colloidal gold solution, and the results are read after twenty-four hours.

After a series of examinations for a period of from one to several weeks, our patients were placed on tryparsamid, and later tryparsamid and mercuric salicylate. During the period of treatment, blood specimens were taken every week and lumbar punctures were made usually at intervals of six weeks. After drug administration and during the period of rest, further examinations of a similar character were made.

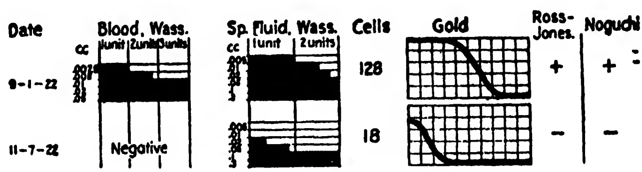

CHART 3. Observations in a case of asymptomatic paresis in a woman, aged 24, who received one course of eight doses of tryparsamid, 3 gm., and eight doses of mercuric salicylate. The second lumbar puncture was made two weeks subsequent to the last treatment. At the beginning of treatment the patient weighed 128 pounds (57 kg.), and at the end, 133 pounds (60 kg.).

Before and during treatment, very complete clinical observations were made, including careful physical and mental examination, blood counts, body weight, etc.

We found intelligence tests (Terman's) to be useful in gaging mental improvement. The test level may not necessarily be fixed by intellectual ability as such, but probably is determined also by such factors as interest on the part of the patient, cooperation, emotional states, and such other mental phenomena as contribute to the final intellectual effort of the patient. In employing such tests it is imperative to adhere to a fixed technic and method, or else the results cannot be compared. Renal functional tests were made in order to exclude kidney injury. During the course of treatment and subsequently, many similar detailed clinical and serologic examinations were made. As a consequence we were able to estimate with fair accuracy the changes that occurred.

At the outset we used tryparsamid without any other antisyphilitic medication. We gave the drug in doses of 5 gm. at intervals of one week over a period of from five to six weeks. The patients were then given no further treatment. It was evident from our initial trial that tryparsamid alone has very marked beneficial effects, both on the clinical and on the serological pictures. After several months under the same clinical observation, it was found that the serologic improvement was not stable. Regression approaching the original findings developed, although clinically the improvement which had occurred seemed stationary. Fearing a clinical relapse, we decided to use

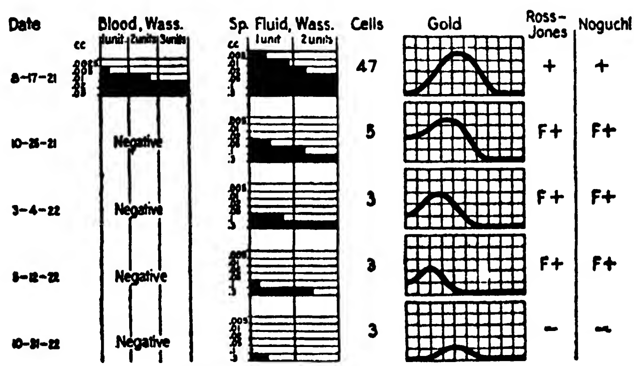

CHART 4. Observations in a case of meningovascular syphilis in which four courses of tryparsamid and mercury were given, each course consisting of eight doses, with rest periods of six weeks. The last lumbar puncture was made one month subsequent to the final treatment. At the beginning of treatment the patient weighed 148 pounds (67 kg.), and at the end, 178 pounds (80 kg.).

mercury in addition to tryparsamid. We then found that the additional use of mercury during the time of tryparsamid treatment resulted in more permanent improvement of the serologic findings as well as apparently more rapid clinical improvement.

When tryparsamid was used in 5 gram doses at weekly intervals, we found that after four or five such administrations, approximately 40 per cent. of our patients complained of dimness of vision. This condition was transient in all except two cases, and disappeared as soon as the drug was stopped. In the two cases in which the conditon was persistent, the patients were far advanced paretics who had had abnormal eyegrounds before treatment. In view of this experience, we

adopted the rule, which we recommend for general application, that every case to be treated with tryparsamid be subjected to careful ophthalmoscopic examination, and that in any case showing retinal changes the drug be used with great caution. Except for the appearance of amblyopia, 5 gram doses were very well tolerated by all. We have given a patient weighing 90 kg. as much as 8 gm. of tryparsamid at one injection without any untoward manifestations. In view of the eye symptoms developing with 5 gram dosage, we began with much smaller doses and gradually increased. By this method, we found that a dose of 3 gm. of tryparsamid, given weekly, yields the therapeutic effect of the drug, and during the period of more than one year we have at no time observed any retinal disturbances or any other untoward manifestations.

Our practice during the last year has been to dissolve 3 gm. of tryparsamid in 10 c.c. of sterile freshly distilled water, and to inject the total amount intravenously. This solution is given at intervals of one week and for a period of eight weeks. At the same time, mercuric salicylate is administered intramuscularly in 1 grain doses. The mercury is given three days before the tryparsamid and a total of nine such injections alternated with the eight injections of tryparsamid comprises a course. It has then been our practice to give the patient a rest period of from five to eight weeks, when a second similar course is repeated. After this second course and a period of rest, if there is continued evidence of activity, or the case is still serologically positive, a third course is given.

Results.

With few exceptions, all of our patients have remained under our clinical observation and a large number have had a complete serologic examination made recently.

Of the forty-two far advanced paretic patients treated, twenty-one have been discharged from the hospitals and are working. In the twelve earlier cases and relatively acute, extremely agitated type of paretic, our results have been most pronounced. Seven patients have fully recovered their normal mentality and have been discharged and are earning their livelihood. The remaining five patients are mentally in condition to earn their livings; but, on account of serologic findings they have not been discharged.

This brings up for consideration a so-called characteristic of paresis which is so frequently offered as an explanation for response to any form of treatment; that is, the so-called "remission." Our experience with paresis in all its stages has been large and spread over a considerable number of years. We cannot, in the light of this experience,

TABLE I.

Summary of Results in Patients Committed as Insane.

Classification.	Clinically.			Blood Wassermann reaction.			Spinal fluid.				
	Arrested and working.	Improved.	Unimproved.	Became negative.	Mildly positive.	Unchanged.	Wassermann.	Cell.	Globulin.	Colloidal gold.	
Paresis, late...... (42 cases)	21	4	17	80%	17%	3%	30%	50%	34%	12%	Negative.
							54%	50%	52%	48%	Reduced.
							16%	—	14%	40%	Unchanged.
Paresis, early..... (12 cases)	7	5	—	84%	16%	—	40%	72%	56%	24%	Negative.
							52%	28%	44%	68%	Reduced.
							8%	—	—	8%	Unchanged.
Taboparesis...... (2 cases)	1	1	—	100%	—	—	50%	100%	50%	50%	Negative.
							50%	—	50%	50%	Reduced.
							—	—	—	—	Unchanged.
Tabes........... (1 case)	1	—	—	100%	—	—	100%	100%	100%	100%	Negative.
							—	—	—	—	Reduced.
							—	—	—	—	Unchanged.
Meningovascular syphilis...... (2 cases)	2	—	—	100%	—	—	50%	100%	50%	50%	Negative.
							50%	—	50%	50%	Reduced.
							—	—	—	—	Unchanged.
Generalized syphilis........ (2 cases)	2	—	—	100%	—	—					
	(Both patients suffering from acute alcoholic psychosis.)										

subscribe to the oft-repeated statements of the frequency of remissions in paresis; that is, if one is to regard as a remission the complete disappearance of all mental symptoms and a return to fairly normal mental activity over a period of six months to one year. Such a definite and decided change in the course of paresis is, in our experience, extremely rare among untreated cases.

Of the total of fifty-four cases of paresis studied, twenty-eight patients have been discharged from the hospitals and are holding positions and earning a livelihood for themselves and their families for periods ranging from six months to two years.

It should be noted that tryparsamid has a definite effect on nutrition. The majority of our patients have made a decided gain in weight and their general state of health has markedly improved. This phase

TABLE II.

Summary of Results in Noncommitted Ambulatory Cases.

Classification.	Clinically.			Blood Wassermann reaction.			Spinal fluid.				
	Arrested.	Improved.	Unimproved.	Became negative.	Mildly positive.	Unchanged.	Wassermann.	Cell.	Globulin.	Colloidal gold.	
Paresis, asymptomatic	13	1	—	84%	16%	—	42%	77%	56%	28%	Negative.
							51%	23%	44%	72%	Reduced.
(14 cases)							7%	—	—	—	Unchanged.
Taboparesis	3	—	—	67%	33%	—	33%	67%	33%	—	Negative.
(3 cases)							67%	33%	67%	67%	Reduced.
							—	—	—	33%	Unchanged.
Tabes.	3	1	—	100%	—	—	25%	75%	25%	—	Negative.
(4 cases)							75%	25%	75%	100%	Reduced.
							—	—	—	—	Unchanged.
Meningovascular	7	1	—	75%	25%	—	37%	50%	50%	37%	Negative.
syphilis (8 cases)							50%	50%	50%	50%	Reduced.
							13%	—	—	13%	Unchanged.
Generalized syphilis (7 cases)	7	—	—	42%	29%	29%					

of the action of the drug cannot be overlooked, especially in patients in a poor state of nutrition. These patients on the average show a gain of weight of about 20 pounds (9 kg.).

Of the ten cases of meningovascular syphilis treated, the blood Wassermann reaction became negative in eight and was mildly positive in two. The spinal fluid serologic findings became negative in four of the cases, improved in five, and was unchanged in one case.

The effect of treatment from the clinical and serologic standpoints is summarized in the accompanying tables. We present four charts to illustrate in detail the effect of treatment on the serologic findings. We found that tryparsamid alone altered the blood Wassermann reaction in more than 80 per cent. of the cases treated. Some cases became absolutely negative, as previously stated. However, when mercury was used, together with tryparsamid, the blood Wassermann reaction was more promptly altered. In nearly every case so treated there was a demonstrable change in the direction of negativity. Some cases were never made completely negative.

The spinal fluid Wassermann reaction was favorably altered in seventy-eight cases. In thirty-two cases the spinal fluid became completely negative—that is, complete hemolysis with 0.5 c.c. of fluid and one unit of available complement. In fifty-four cases the lymphocyte count was reduced to normal range. Forty cases showed negative globulin tests after treatment. The colloidal gold test was favorably affected in sixty-eight cases. The changes in this reaction followed the order frequently observed when syphilis of the central nervous system is effectively treated. The initial paretic curves would change and become more of the meningovascular type. Eventually this response would be changed, and in eighteen cases the reaction became negative. Of the total number of cases treated in which there were completely positive serologic findings in the spinal fluid, eighteen became negative in all phases of spinal fluid serology.

From our present experience we recommend this method of treatment in properly selected cases: The tryparsamid should be used in doses of 3 gm., dissolved in sterile freshly distilled water, sufficient to make approximately a 30 per cent. solution; that is 3 gm. dissolved in 10 c.c. of water. This solution is given intravenously at intervals of one week and for a period of eight weeks. At the same time, mercuric salicylate should be administered intramuscularly in 1 grain doses. The mercury should be given three days before the tryparsamid, and a total of nine such injections with eight of the tryparsamid should comprise a course. After such a course has been given, we believe that a rest period of from five to eight weeks is good practice, when a second course similar to the first is repeated. After the second course and after a period of rest, if there is still evidence of

clinical activity or the case is still serologically positive, a third course should be given.

We have had four patients on weekly injections of 3 gm. of this drug over a period of six months without a rest period, during which time careful kidney function tests were made, and at no time was there any evidence of renal derangement.

From our present experience we recommend the use of tryparsamid and mercury in cases of neurosyphilis and believe that this combination is more effective in paresis than any other measures now in use. The beneficial effects are especially striking in early paresis. We also recommend that this drug combination can be used advantageously with patients that cannot take the other arsenicals used in the treatment of syphilis. This combination is also recommended for trial in cases that are Wassermann fast. Owing to the absence of any untoward reactions and the convicton that this method of treatment is less drastic than arsphenamin and neo-arsphenamin, we recommend its use in cases of late syphilis in patients past middle age. Generally, this type of case must be more gently handled than younger patients, and we believe that this combination can be advantageously used for patients that might be very seriously damaged by arsphenamin.

Finally, we recommend the use of these drugs in cases exhibiting a poor state of nutrition, as tryparsamid has a definitely favorable effect on the nutrition.

In the use of tryparsamid it is imperative that a very complete physical examination be made, especially of the eyegrounds. Furthermore, that any case under treatment should be watched carefully for retinal changes and the patient so questioned as to bring out any disturbance in vision. It is, of course, important not to suggest symptoms to the patient; but if there is any evidence of a visual disturbance, the drug should be immediately withheld.

SUMMARY.

Tryparsamid and mercuric salicylate, given according to the method herein described, is especially effective in early paresis and other forms of neurosyphilis. In our experience, it is more effective than any other form of treatment used.

We also recommend its trial in cases that are Wassermann fast and in syphilitic patients showing a poor state of nutrition and those beyond middle age.

Tryparsamid, when employed in dosage of 3 gm., produces no local or general symptoms, either immediate or late, and can be used to advantage in cases in which the patient cannot tolerate other arsenicals.

Clinical and serologic improvement in early and violent cases of paresis is extremely striking. The recovery or improvement is not absolutely stable; but the use of mercuric salicylate with the tryparsamid tends to stabilize the improvement. Some of the cases, after a period of two years, again show serologic activity, although clinical improvement has continued without change. The attitude of the patient toward this entire method of treatment is such that continued treatment and repeated courses are taken without complaint and, indeed, with cooperation.

Finally, we desire to emphasize the fact that tryparsamid possesses the potentiality of injuring the optic tract and should not be used in cases showing degenerative changes in the retina.

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THE RELATION BETWEEN BLOOD DESTRUCTION AND THE OUTPUT OF BILE PIGMENT.

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Many facts point to a close relation between blood destruction and the yield of pigment in the bile. The assumptions currently made in this connection by physiologists and clinicians have recently been summarized by one of us and critically reviewed.[1] A method now available for total bile collection[2] renders possible an experimental test of some of them.

The Pigment Changes Following Bile Loss.

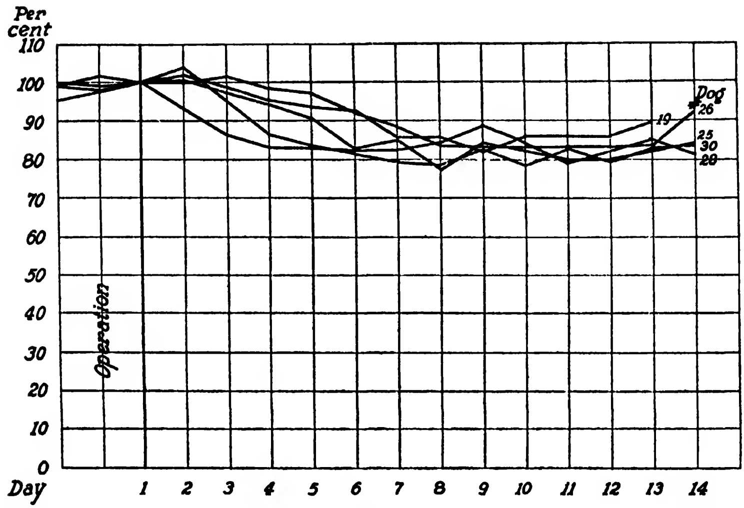
In dogs losing all their bile, significant changes take place in the hemoglobin percentage of the blood and in the bilirubin content of the secretion lost. They have been briefly described in a previous paper in which the methods employed for the care of the animals and the study of the bile have also been outlined.[3] As a rule, for several days after the operation whereby drainage of the common duct is effected, the hemoglobin percentage alters but little (Text-figs. 1 and 2), though in general it tends to fall slowly to about four-fifths of the initial value in the course of a week (Text-fig. 1). The bilirubin output by contrast falls off briskly from the beginning, and at the end of the same period of time averages only about half of the first amount (Text-fig. 3).[4] There can be no doubt that the large early amount is sometimes derived in part from extravasated blood. The yield from dogs in which hemorrhage into the tissues was known to

[1] Rous, P., *Physiol. Rev.*, 1923, iii, 75.

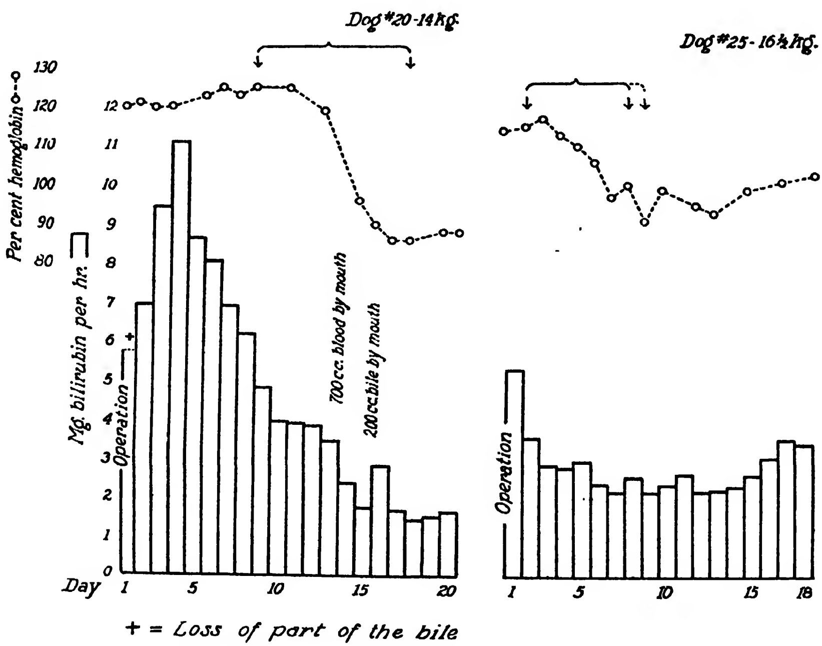
[2] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.

[3] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

[4] See also McMaster, Broun, and Rous,[3] Text-fig. 2. Several instances complicated by blood extravasation are there included.

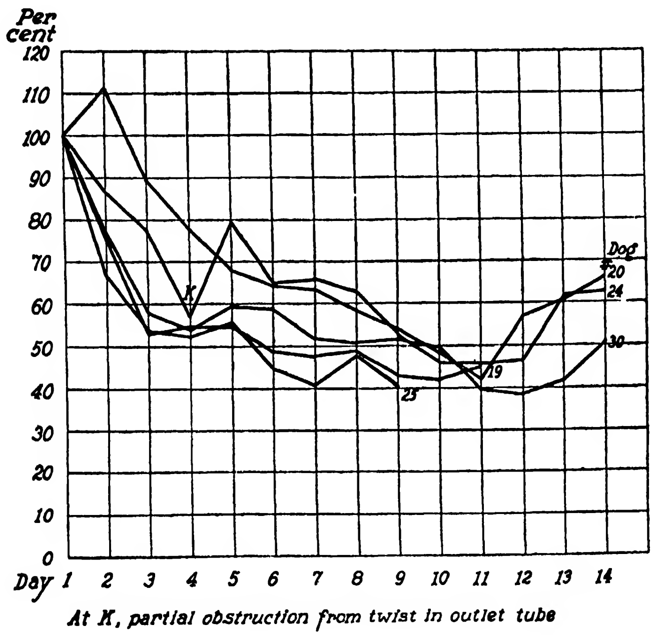


TEXT-FIG. 1. Changes in the hemoglobin percentage expressed in terms of the finding on the 1st day after operation.—Uncomplicated instances.



TEXT-FIG. 2. (See Table I.) Unusually marked postoperative changes in the hemoglobin percentage and bilirubin output. The periods dealt with in Table I, during which the hemoglobin percentage fell rapidly, are included between the bracketed arrows on the chart

have taken place increased markedly during the period when the hematoma was breaking down (as, for example, in Dog 30 of Text-fig. 6). Yet when careful hemostasis was practised, and at most only a few cubic centimeters of blood left the vessels, the pigment yield halved in from 7 to 10 days (Text-fig. 3).

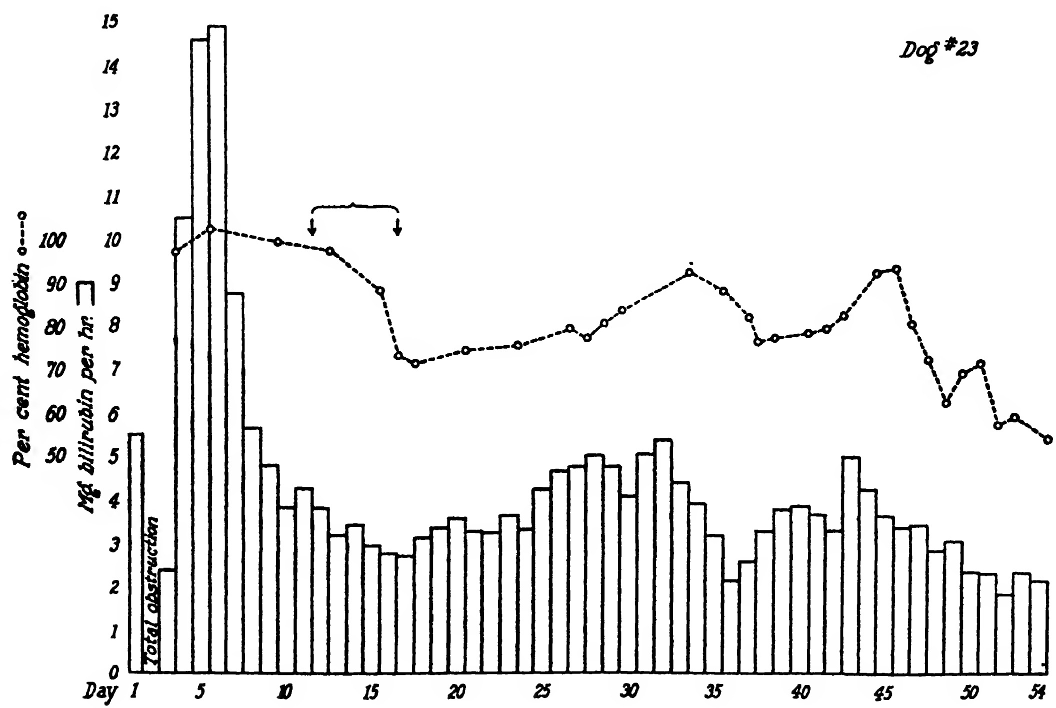


TEXT-FIG. 3. Postoperative changes in the bilirubin output in uncomplicated instances, as expressed in percentages of the yield of the 1st day.

There has been no general recognition of these postoperative changes for the excellent reason that animals in which a bile fistula has been established after the classical fashion are ordinarily not worked with until wound healing has taken place. Brugsch and Retzlaff[5] alone appear to have noted them, remarking as an aside to other matters that during the first 14 days of bile loss there is a falling off in number of red cells and bilirubin output. They furnish no figures on the point. Their dogs were suspended in slings and all the bile was collected.

The later pigment changes vary with the individual. In our animals they were unquestionably affected by certain of the experi-

[5] Brugsch, T., and Retzlaff, K., *Z. exp. Path. u. Therap.*, 1912, xi, 508.



TEXT-FIG. 4. (See Table I.) Concurrent changes in the hemoglobin percentage and the bilirubin output during the course of a marked progressive anemia. The periods dealt with in Table I, during which the hemoglobin percentage fell rapidly, are included between the bracketed arrows on the chart.

mental procedures employed, notably by exercise[6] and by bile feedings.[6] But always in uncomplicated instances, the anemia indicated by the early drop in the hemoglobin percentage persisted, though varied, at times, by intervals of more or less complete recovery.[7] Usually it remained slight. We are inclined to believe that in the two animals in which it became marked (see Text-fig. 4; also McMaster, Broun, and Rous[8]), there was another factor involved besides loss of the bile. Dog 23 of Text-fig. 4 of the present work had a severe intercurrent bronchitis, and later a gradually developing intestinal obstruction; while Dog 24 of the previous paper[8] occasionally lost blood from hemorrhoids. With mild anemia the hemoglobin percentage as a rule altered but slightly from week to week, as did the bilirubin output also.[7] Every well defined blood change found reflection in a change in this last. In the two dogs just mentioned, with pronounced anemia, remissions occurred at frequent intervals, the hemoglobin percentage rising for a time only to fall again, usually to a lower level; and as it rose and fell so too did the yield of bilirubin. The subjective element can be ruled from account in connection with the findings; for the estimations on blood and bile were made by different workers each unaware of the other's results, while, furthermore, the remarkable correspondence in the pigment changes did not come to attention until the data were correlated, weeks after they had been obtained. The synchronous alterations were slow and wave-like, often as much as a fortnight elapsing between crest and crest. Circumstances have prevented us from following them for more than 3 months.

The character of the anemia was repeatedly studied. Always it was of secondary type, as shown by cell counts and the examination of stained smears. The color index was somewhat, but not greatly, less than the normal; poikilocytosis, though present, was never marked. The number of circulating reticulated cells, which was followed by Robertson's[9] technique, proved to be even less than in healthy dogs.[10]

[6] Broun, G. O., McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 699.

[7] McMaster, Broun, and Rous,[3] Text-fig. 5.

[8] McMaster, Broun, and Rous,[3] Text-fig. 4.

[9] Robertson, O. H., *J. Exp. Med.*, 1917, xxvi, 221.

[10] Krumbhaar, E. B., *J. Lab. and Clin. Med.*, 1922, viii, 11.

The inference seems justified that the hematopoietic tissue was less active than usual. Certainly it was putting forth cells somewhat deficient in pigment.

The charts leave no room for doubt that a quantitative relationship of some sort exists between the blood and bile pigments. But is it the relationship generally supposed? The calculations in vogue nowadays on blood destruction as expressed in the yield of bilirubin or urobilin have for basis the assumptions that from the hemoglobin molecule 4 per cent by weight of hematin is derived, and that for every gram of hematin approximately 1 gm. of bilirubin is excreted.[11] Do the data of our experiments accord with such a view?

Stadelmann[12] long ago noted that after injections of hemoglobin the expected rise in the bilirubin content of the bile was incompletely realized. His finding seems to have been wholly lost sight of. Recently Whipple has sponsored the conception that bilirubin has other sources besides the blood,[13] a principal one being the carbohydrates of the food. We have corroborated the observations of Whipple and Hooper[14] on the effects of carbohydrate diet to alter the bilirubin output transiently but have shown that the alteration is referable merely to a changed rate of pigment evacuation, not to a change in the amount manufactured from day to day, which remains unaffected.[15] Whipple and Hooper collected the bile during 6 or 8 hours of each 24. They made interesting observations on the pigment excreted during this time, following injections of the hemoglobin of laked blood;[16] but the circumstances of the work were such that they could reach no conclusion on quantitative relationships. They noted, however, that in anemic animals the bilirubin output for the 6 hour period was sometimes astonishingly small.

The Calculated Pigment Relationships.

As already stated, every considerable drop in the hemoglobin percentage of our dogs, uninduced by extraneous means, was accompanied by a drop in the bilirubin yield. Granting that a lessened hemoglobin

[11] Abderhalden, E., Lehrbuch der physiologischen Chemie, Berlin and Vienna, 4th edition, 1920, i, 746.

[12] Stadelmann, E., Der Icterus und seine verschiedenen Formen. Nebst Beiträgen zur Physiologie und Pathologie der Gallensecretion, Stuttgart, 1891, 40.

[13] Whipple, G. H., *Physiol. Rev.*, 1922, ii, 440.

[14] Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1916, xl, 349.

[15] Rous, P., Broun, G. O., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 421.

[16] Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1917, xliii, 258.

percentage means a proportionately lessened amount of the pigment in circulation, one can gain an idea from the data as to whether the known amount of bilirubin excreted during the period in which the blood pigment apparently underwent a lessening represents accurately the amount of blood withdrawn from circulation, or, to speak loosely, destroyed. In Dog 20, for example, the hemoglobin percentage fell from 125 per cent to 86 per cent in the course of 10 days. Does this change find exact expression in the bilirubin output?

Several potential sources of error or complication must be considered prior to an analysis along the lines indicated. Alterations in the hemoglobin percentage are known to be only roughly indicative of changes in the total amount of the pigment. And may not a part of the bilirubin from blood suddenly destroyed be excreted so slowly by the liver as to escape reckoning? The charts furnish evidence on the point. They show that whenever there was a sharp decline in the hemoglobin percentage an equally sharp decline in the bilirubin output took place, both reaching their lowest point at the same time. This could not have happened had bilirubin excretion been notably delayed. The possibility that the substance has other sources besides hemoglobin can, in the present relation, be dismissed. If it has such sources these would appear to be negligible in the present relation, as witness the charts. Changes in the diet influence only the rate of pigment evacuation, and this transiently. But exercise, by increasing the rate of blood destruction, may act to increase markedly the total yield.[3] To minimize this disturbing influence the animals were confined in metabolism cages. Noteworthy errors in bile collection and in bilirubin quantitation were ruled out by the methods employed; and only such figures on hemoglobin percentage have been admitted to consideration as were corroborated by the findings of the preceding or subsequent 24 hours. The factor of blood losses masked by replacement must have influenced the results, sometimes, doubtless, to an important extent. For during the periods supplying the data under analysis, in which the hemoglobin percentage fell, there must have been some repair of the blood loss, since the formation of corpuscles can scarcely have ceased altogether. But for present purposes it will be simplest to suppose that it did cease, and that in consequence the fall in hemoglobin represents the actual blood loss, not, as was actually the case, a somewhat greater loss compensated in part by replacement.

In Table I the actual bilirubin output is contrasted with the expected output during four periods (indicated by brackets and arrows on Text-figs. 2 and 4), in which the hemoglobin percentage lessened rapidly and markedly, without loss from the body of blood, bile pigment, or urobilin, in the stools or urine. Dog 23 of Text-fig. 4 was, like the other animals, in excellent condition at the time, giving no sign of the complicating maladies which rendered its later condition poor. The data on Dog 24 to be found in a previous paper[7] have not been tabulated

TABLE I.

Bilirubin Output during an Intercurrent Fall in the Hemoglobin Percentage (Text-Figs. 2 and 4).

Dog No.	Weight.	Period included.	Calculated blood amount.	Hemoglobin.				Bilirubin output.		Remarks.
				First.	Last.	Drop in hemoglobin.		Ex-pected.	Actual.	
	kg.	*days*	*cc.*	*per cent*	*per cent*	*per cent*	*gm.*	*gm.*	*gm.*	
23	$14\frac{1}{2}$	5	1,334	98	73	25	46.1	1.84	0.42	Bile sterile. No bilirubinuria.
25 a	16	6	1,471	115	100	15	32.3	1.29	0.38	" " " "
25 b		7			91	24	48.8	1.95	0.46	" " " "
20	$12\frac{3}{4}$	10	1,172	125	86	39	63.2	2.53	0.72	" " " "

because the animal occasionally bled from hemorrhoids. The losses in total hemoglobin have been calculated from the percentage changes, on the basis that 9.2 per cent of the body weight of the animal consists of blood;[17] that a blood having 100 per cent of hemoglobin by the Newcomer standard we employed has 13.8 per cent of hemoglobin in every 100 cc.; and that hemoglobin yields 4 per cent by weight of hematin.[18] Most authors assume that hematin appears weight for weight as bile pigment, but in the calculations we have preferred to adopt the conservative estimate of Eppinger and Charnas[19] that for every gram of hematin 0.9 gm. of bilirubin is elaborated.

As the four tabulated instances show (Table I), the actual bilirubin output during periods of blood destruction came to but a fraction of the expected output, averaging about one-third of it, despite the circumstance that several elements in our calculations combined to give a minimal figure for this latter. Corroboratory examples could be culled from our unpublished data.

The Actual Pigment Relationships.

Experiments were now begun to measure the amount of blood destruction more exactly. Advantage was taken of the regularity with which anemia follows upon bile loss, and the total hemoglobin of several animals was ascertained 24 hours prior to intubation of the common duct, and again some days later when the hemoglobin percentage had dropped (Text-fig. 5). The decrease in circulating pigment thus measured must be referred in part to hemorrhage at operation; and though, owing to hemostasis, such part was negligible, it introduces an element of uncertainty into the data. For this reason some later estimates of total hemoglobin were made, to be compared with the findings a few days after operation. But in only one of three instances had any demonstrable loss of hemoglobin occurred in the interval (Text-fig. 5). In order to provide a destruction of corpuscles for the purpose of the observations, recourse was ultimately had to treadmill exercise,[20] and to the transfusion of alien blood.

[17] Smith, H. P., Arnold, H. R., and Whipple, G. H., *Am. J. Physiol.*, 1921, lvi, 336.

[18] The figure for the standard was determined by Dr. J. M. Neill of the Hospital of The Rockefeller Institute, who kindly carried out for us analyses of the pigment content of specimens of dog blood.

[19] Eppinger, H., and Charnas, D., *Z. klin. Med.*, 1913, lxxviii, 387.

[20] Broun, G. O., *J. Exp. Med.*, 1922, xxxvi, 481: 1923, xxxvii, 113.

In Tables II and III and Text-figs. 5 and 6, the results of all the experiments find place. The dogs had, without exception, the appearance of vigorous health. They were fed for the most part on a mixture of bread and cooked lean meat in fixed proportion, but occasionally received bread and milk, or raw lean meat. The stools, which were clay-colored, were regularly examined for blood (guaiac reaction) and for stercobilin (Schlesinger's test). Several animals that had been destined for the work were discarded because one or both of the pigments was found. Neither occurred in any of the animals employed. The urine, daily searched for urobilin and urobilinogen and hematoporphyrin, was regularly devoid of the first mentioned substances and of abnormal amounts of the last. Whenever bilirubinuria was sufficiently pronounced for quantitation, the amount of bile pigment escaping in this way was determined according to a procedure already described.[21] Such determinations were necessary in but one animal, Dog 19 (Text-fig. 6). Whenever the bile became infected, incubation tests were carried out to find whether the organisms altered bile pigment. This they never did, nor did they prove pathogenic for the animal harboring them. The dogs were the last of a considerable series intubated for bile collection and fared best of all, the laparotomy wound healing by first intention in practically every case. They were sacrificed after from 1 to 3 months of observation, sometimes because of the development of total obstruction from stone in the common duct, and again for reasons of expediency.

The method of Van Slyke and Salvesen[22] was employed for the determination of total hemoglobin, and by one of us familiarized with it through much previous work.[20] The routine observations on the hemoglobin percentage were made by another individual, and it has been reassuring to observe,—as bearing out an assumption upon which Table I is based,—how nearly the percentage changes in most instances correspond with those in the total pigment. To arrive at a figure on the expected output of bilirubin, the current view on the quantitative relationship between hemoglobin and this pigment was adopted, as it had been for Table I. And, as in the case of this table, the influence of the hematopoietic activities to mask the amount of blood destruction, making it appear less than it actually is, has been left from account. In some instances there was a 24 hour period prior to operation during which, of course, no bile could be collected. The actual bilirubin output for the time can only be conjectured. We have supposed it to equal the largest amount subsequently obtained in the same period of time.

Intercurrent Losses of Total Hemoglobin (Table II, Text-Fig. 5).—In Dog 26 the loss in total hemoglobin during the period of the 6 days following operation, together with the 24 hours immediately preceding it, would seem to have been extremely large,—equivalent to that in

[21] Haessler, H., Rous, P., and Broun, G. O., *J. Exp. Med.*, 1922, xxxv, 533.

[22] Van Slyke, D. D., and Salvesen, H. A., *J. Biol. Chem.*, 1919, xl, 103.

about 500 cc. of whole blood,—despite the fact that hemostasis was nearly perfect. The actual output of bilirubin proved to be less than a third of that warranted by such blood destruction. During the next 6 days the loss of blood was less, though still considerable. It was almost exactly indicated by the changes in the hemoglobin percentage, which had not been the case at first. The actual output of bilirubin now came to one-third of the expected output.

Dog 30 lost during 5 days, inclusive of that before operation, an amount of hemoglobin such as had existed in about 300 cc. of the whole blood. The actual bilirubin yield for the period totalled 43 per cent of the expected quantity. When the total hemoglobin was next determined 10 days later, a more moderate loss in it was found. The actual bilirubin output now slightly exceeded the expected one. It goes without saying that the smaller a hemoglobin loss, and the greater the period of time over which it is distributed, the more important will the factor of cell replacement be, as masking the amount of destruction. Gradual losses may be wholly concealed by replacement. When this is the case the daily bilirubin yield will be considerable, although, since the hemoglobin percentage does not alter, none whatever would be expected under the limiting conditions of our calculation in which the element of replacement is ignored. The findings in the second period of Dog 30, in which the actual bilirubin output exceeded the calculated figure, afford a partial illustration of these facts. They are more strikingly evident in the data on Dog 28.

The hemoglobin percentage of Dog 28 fell abruptly during an initial period of 5 days without any noteworthy diminution in the total hemoglobin. The actual bilirubin output for the period many times exceeded the expected one. An error in the first estimate of total hemoglobin may be invoked to explain the anomalous results, and the discrepancy between the changes in percentage of the pigment and its total quantity point to such a probability. But if the claim be allowed, a similar one must be lodged against the findings for the first period of Dog 26, in which a discrepancy of almost as great dimensions, but opposite in direction, was observed.

During the second period of Dog 28 there was almost no change in the percentage and total quantity of the blood pigment. Whatever the daily loss of cells may have been it was practically concealed by

TABLE II.

Bilirubin Output during Intercurrent Changes in Total Hemoglobin (Text-Fig. 5).

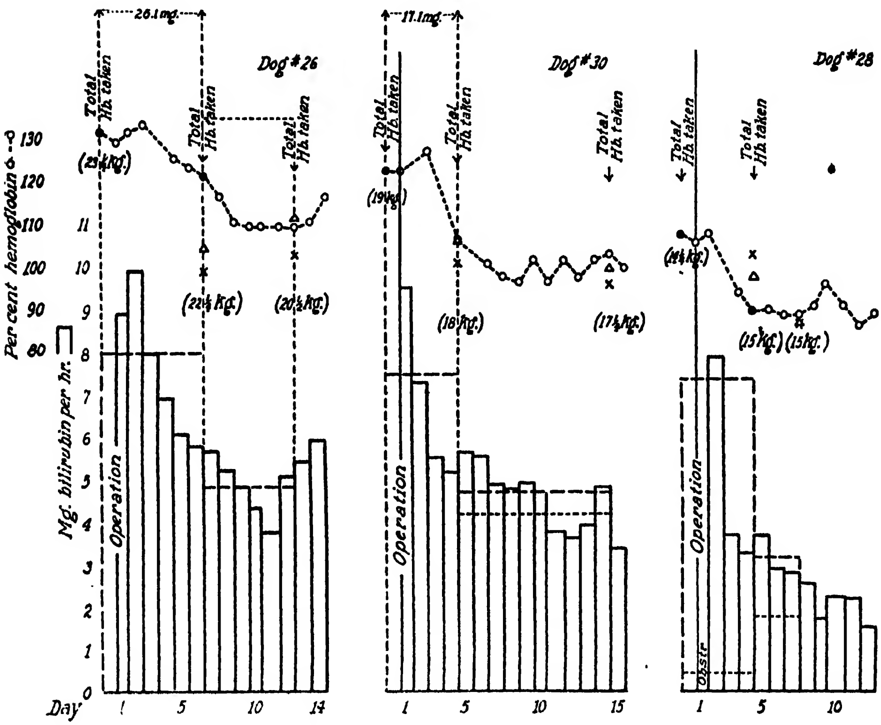
Dog No.	Weight.	Elapsed period.	Cell volume.	Plasma volume.	Blood volume.	Hemoglobin per 100 cc. of blood.	Total hemoglobin.	Loss of hemoglobin within the body.	Bilirubin output for period. Expected.	Bilirubin output for period. Actual.	Remarks.
	kg.	*days*	*cc.*	*cc.*	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
26											Total hemoglobin determined on the day previous to operation.
	$23\frac{1}{2}$		1,121	968	2,089	21.12	441.5				
	$22\frac{1}{4}$	7	1,007	915	1,922	17.2	330.5	105.05	4.38	1.33	After 5 days of bile collection.
	$22\frac{1}{4}$						330.5				
	$20\frac{1}{2}$	6	941	837	1,778	15.7	279.0	46.49	1.94	0.69	12th day of bile collection. Bile infected after the 8th day. No bilirubinuria.
30											Total hemoglobin determined on the day previous to operation.
	19		990	935	1,925	16.45	316.2				
	18	5	826	925	1,755	14.81	260.0	51.43	2.05	0.88	After 4 days of bile collection.
	18						260.0				
	$17\frac{1}{4}$	10	795	904	1,709	13.67	233.3	22.37	0.90	1.1	13th day after operation. Bile sterile throughout.

28	14½		559	619	1,178	14.83	174.8				Total hemoglobin determined on the day previous to operation.
	15	5	569	642	1,201	13.95	167.8	2.63	0.11	0.73	After 4 days of bile collection.
	15						167.8				
	15	3	543.5	746.5	1,290	12.49	161.0	2.76	0.11	0.22	After 7 days of bile collection. Bile sterile throughout.

* As distinct from the loss incurred in the determinations.

No bilirubinuria in any animal except No. 30, in which an occasional trace was found, as in many normal animals.

replacement. Under such circumstances the actual output of bilirubin could not but greatly exceed the calculated one, as was indeed the case.



X = Hemoglobin per cent as calculated from the reduction in total hemoglobin.
Δ = Hemoglobin per cent as calculated from the reduction in total hemoglobin and corrected for change in body weight.
— — — = Actual bilirubin output per hour during the period.
-------- = Expected output.

Text-Fig. 5. (See Table II.) The actual and the calculated bilirubin yields during periods of intercurrent losses of total hemoglobin.

Induced Losses of Total Hemoglobin (Table III, Text-Fig. 6).—Two dogs (Nos. 28 and 30) employed for the work just discussed were exercised some weeks later on a treadmill for 3 to 5 hours on each of 4 successive days, in order to bring about a rapid destruction of red cells. Experience had shown that exercise of the sort could not be

begun immediately after a determination of total hemoglobin by the CO method without great risk of hemorrhage into the tissues through the puncture openings in the jugular veins; while, furthermore, the dogs were not fitted to run well until all the hemoglobin was once again in a condition to combine with oxygen. For these reasons the total blood pigment was ascertained 18 to 24 hours prior to use of the treadmill. Directly after the animal was taken from the mill on the 4th day, the total hemoglobin was again measured, although the 24 hour period of bile collection which included the final exercise hours did not come to an end until the next morning. It follows that the observations upon the blood were not wholly synchronous with those upon the bile but preceded the latter by about 18 hours on the average. Whether one ignore this fact or make allowance for it in computing the actual bilirubin output for the days of exercise,—and we have done both in Table III,—the result is the same. The actual bilirubin output of the animals amounted to little more than half of the expected one.

The destruction of compatible blood transfused to dogs is known to begin early and to proceed rapidly. The course of events in Dog 19, which was somewhat anemic when given 328 cc. of citrated dog blood containing 51.5 gm. of hemoglobin, well illustrates the rule. The concurrent alterations in hemoglobin quantity and bilirubin output proved highly instructive. In a first period of 2 days, following introduction of the strange blood, there was a considerable destruction of cells, as shown by the alterations in total hemoglobin, though without bilirubinuria or symptoms. The increase in the yield of bile pigment was many times less than it should have been on calculation. Blood destruction proceeded more gradually during the next 7 days, as evidenced both by the day to day reductions in the per cent of hemoglobin and the changes in total amount. Bilirubinuria was still absent, and the general condition of the dog excellent. The actual yield of bile pigment for this period was not greatly less than the expected. Now the state of affairs altered almost over night. The hemoglobin percentage began to fall more rapidly, there was pronounced bilirubinuria, and an anemia developed more marked than that prior to transfusion. There was no tissue icterus, but the animal appeared languid. The causes for the developing anemia have been

TABLE III.

Bilirubin Output during the Course of an Induced Reduction in Total Hemoglobin (Text-Fig. 6).

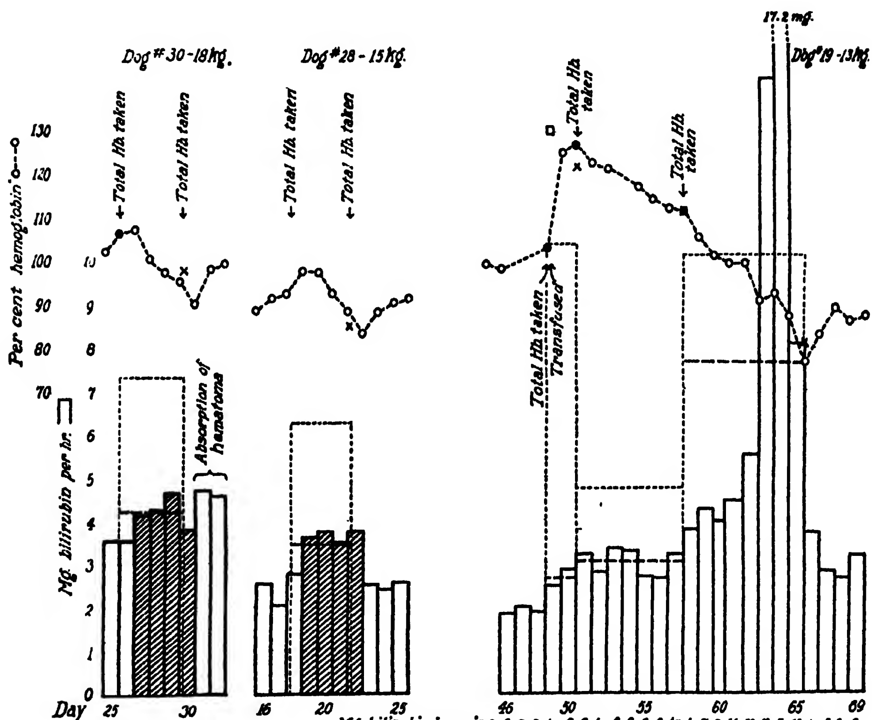
Dog No.	Weight.	Treatment.	Elapsed period.	Cell volume.	Plasma volume.	Blood volume.	Hemoglobin per 100 cc. of blood.	Total hemoglobin.	Loss of hemoglobin within the body.*	Bilirubin output for period. Expected.	Bilirubin output for period. Actual.	Remarks.
	kg.		*days*	*cc.*	*cc.*	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
30	18	5 hrs. of treadmill exercise on each day except the last, when 3 hrs. only was given.	4	821 765	1,024 1,185	1,845 1,950	13.95 12.10	257.4 236.0	17.4	0.7	0.4 (0.4)†	The total hemoglobin was determined 22 hrs. before beginning exercise, and again 3 hrs. after its close. No bilirubinuria. Bile sterile.
28	$14\frac{3}{4}$	5 hrs. of treadmill exercise on each day except the last, when 4 hrs. only was given.	4	531 511.5	669 704:5	1,200 1,216	14.1 12.35	169.2 150.1	15.05	0.6	0.33 (0.35)†	The total hemoglobin was determined 22 hrs. before beginning exercise, and again 3 hrs. after its close. No bilirubinuria. Bile infected.

	13	Transfused.		564	598	1,162	15.8	183.6				The total hemoglobin was determined 2 hrs. before transfusion.
			Calculated amount of hemoglobin after transfusion 232.1 gm.									
			2	656	483	1,139	19.11	217.6	12.58	0.5	0.11	No bilirubinuria. Bile infected.
19	13¼		7	602	532	1,134	16.94	217.6 192.2	19.89	0.8	0.505	No bilirubinuria.
			8	492	706	1,198	11.58	192.2 138.9	48.75	1.95	1.57	Marked bilirubinuria (the 85 mg. of pigment excreted during the period are included in the actual bilirubin output).

* As distinct from the loss incurred in the determinations.

† Total bilirubin output during the four 24 hour periods of bile collection which cover the days on which the dogs were exercised.

dealt with by Robertson.[9] Prominent among them is marrow inactivity induced by the plethora after transfusion. Because of the temporary lack of need for new red cells, there occurs almost no elaboration of them by the animal while the plethora lasts, and when the strange blood is suddenly made away with through the action of induced antibodies, anemia inevitably develops because the residuum of host corpuscles is small. The expected bilirubin output during



X = Hemoglobin per cent as calculated from the reduction in total hemoglobin.

□ = Hemoglobin per cent as calculated from the amount of hemoglobin added by transfusion.

— — — = Actual bilirubin output per hour during the period.

-------- = Expected output.

TEXT-FIG. 6. (See Table III.) The actual and the calculated bilirubin yields during periods of induced loss of total hemoglobin. The actual output on the days of exercise finds record in the cross-hatched columns.

this period of rapid destruction in Dog 19 was large, more than twice that of the days immediately preceding, yet the actual output came nearer to equalling it than previously. Thereafter an abrupt turn for the better in the general condition was noted, the bilirubinuria ceased, the bile became much less pigmented, and recovery from the anemia began. The total amount of hemoglobin was not ascertained again.

DISCUSSION.

In the analysis (Tables II and III) of the data from the last two sets of experiments here discussed, the actual bilirubin output has been contrasted with the expected one which was estimated from the known loss in total hemoglobin as if so much pigment in the form of blood had been taken from a jar and bilirubin derived from it by chemical means. As already mentioned, losses masked by replacement through the hematopoietic activities were disregarded, and of necessity, since they could not be gauged. Despite their potential influence to increase the bilirubin output, this was always less, usually several times less, than the expected quantity whenever a marked and rapid loss of the blood pigment was demonstrable.

These observations show clearly that the quantitative relation existing between blood destruction and the bilirubin output is very different from that generally supposed. Save under special circumstances, which themselves go to prove the rule, the yield of bile pigment was far below what it should have been, to judge from the amount of hemoglobin removed from the circulation. Under the special circumstances referred to, when the body was oversupplied for the time being with the products of blood destruction, as shown by the state of the urine, the actual yield of bilirubin approached the calculated one. Usually the actual yield was less than half of this latter, which was computed on a basis that yielded a minimal figure for it.

Why, in our experiments, did the actual yield of bilirubin fall so far short of the expected one? The quantitative relationships between hemoglobin, hematin, and bilirubin, as such, can scarcely be impeached. Nor do the charts afford indications of any delay in the excretion of bile pigment. Other sources of it there may be besides the blood, but, as has been said, these could have no influence under the conditions of the work. The explanation evidently is that a

part of the pigment from destroyed blood, out of which bilirubin can be formed, undergoes retention within the body,—a possibility suggested by Stadelmann many years ago.[12] That the iron-containing derivatives of the hemoglobin molecule are held for future use has been generally acknowledged. Bilirubin has been thought of as waste stuff. But recently we have brought evidence that some of it is reabsorbed after reaching the intestine.[6] Whether there is a salvage of material by this means for later use in the manufacture of new red cells remains to be determined. But the blood and the bile changes which follow upon loss of the bile must be discussed in the light of such a possibility.

An immediate question in connection with the postoperative blood changes recorded in the present paper is whether they are referable to the loss of pigment in the bile. No other cause for them was evident if one except a not infrequent reddening of the duodenum, unaccompanied by hemorrhage and consequent doubtless on the absence of bile. Anemia developed in every one of eleven animals studied with special reference to the point, most of them extremely vigorous and all kept under conditions and on a diet which suffice in normal dogs for the maintenance of an excellent blood state. The hemoglobin percentage of Whipple and Hooper's long tended fistula dogs remained consistently high.[13] The observations of these authors appear to have been begun a fortnight or more after operation, when the fistula wound had healed. They attribute the well-being of their dogs to liver feedings or the intermittent passage of a little bile into the intestine through some narrow channel in the tissues. The animals had access to the external fistula opening during 16 or 18 hours of each 24 and may well have licked up some of the escaping bile,—though that they obtained much of it seems unlikely.[23] Bile deprivation in our dogs was complete. Furthermore, ours were kept caged because of the influence of exercise upon the bilirubin output, whereas Whipple and Hooper's were allowed to run in a yard daily. Exercise, as Broun has shown,[20] stimulates the hematopoietic tissue. Possibly it is responsible for alterations in hemoglobin metabolism that are sufficient to counteract the tendency to anemia after bile loss. How-

[23] Foster, M. G., Hooper, C. W., and Whipple, G. H., *J. Biol. Chem.*, 1919, xxxviii, 367.

ever this be, the present findings leave no doubt that such a tendency exists; and the demonstrated activity of the body to conserve bile pigment, together with its ability to retrieve a portion of that excreted into the intestine, give reason for the belief that the anemia results from bilirubin loss. Human beings with a bile fistula frequently develop anemia—in one case reported by Balfour and Ross[24] the hemoglobin had fallen to 30 per cent after 3 years of bile loss, although the physical condition of the individual was otherwise good. But many patients losing all the bile fail rapidly and are the subject of complicating disturbances to which such blood changes as occur may well be referable.

The rapid falling off in the bilirubin output during the 1st week after operation, or, to put the case more directly, the high postoperative yield as compared with the later one, is in some instances clearly the result in part of the destruction of red cells extravasated at operation, as we have pointed out. Possibly some corpuscles are injured by the anesthetic. In any event, the destruction within the body of but a few extra cubic centimeters of blood would suffice, according to current views, to swell markedly the bilirubin total of a 24 hour period. But there is another potential reason for the high output of the 1st day after operation. Under normal circumstances the bilirubin yield by the liver may represent not merely excretion of the pigment but an enterohepatic circulation of it.[6] With the sudden elimination of the latter factor, on intubation of the animal, the biilirubin yield should fall off.

The postoperative anemia in intubated dogs,—one feature of which is a low color index of the cells,—develops a little later and more slowly than the fall in the bilirubin output. It can be explained, as has just been indicated, by the continued loss of such part of the bile pigment as would normally be saved from the intestine by reabsorption and utilized in the formation of new corpuscles. In this relation the evidence in our experiments for differing degrees of conservation of the pigment from destroyed blood has significance. In the transfused dog, No. 19 (Table III, Text-fig. 6), which received alien blood when itself anemic, almost all the pigment derived from

[24] Balfour, D. C., and Ross, J. W., *Arch. Surg.*, 1921, iii, 582.

the destruction of the 2 days immediately following transfusion was retained, to judge from the bilirubin output. During the next period, with a continuance of blood destruction, the actual yield of bile pigment came closer to the expected one; and in a final period, when the latter was large, approximated it still more nearly. The course of events was exactly what one would have anticipated had the body at first a need for the pigment, hence retaining most of it, and then gradually been supplied with as much and more than was required. In line with this idea of a conserving mechanism, which may on occasion be active in saving pigment or again lack occasion to function, is the fact that in those of our animals in which blood destruction was precipitated by exercise or transfusion, the actual yield of pigment in the bile more nearly approximated the expected one than when the reduction in hemoglobin was not artificially induced. The conditions may well have been less favorable to pigment conservation in the first mentioned series of instances. Whipple and Hooper record experiments showing that in anemic dogs given injections of small quantities of hemoglobin, the bilirubin increase during the next 6 hours was far less than in similarly treated non-anemic animals.[16] They did not follow the changes to completion, but their protocols suggest a pigment conservation in the presence of body need. There was no evidence for this in the few protocols of Brugsch and Yoshimoto,[25] who concluded that injected hematin was quantitatively represented in the bilirubin output. But much, obviously, will depend on the immediate body need and the amount of pigment given. Brugsch and Retzlaff injected it on many consecutive days, and lumped their findings for the period.

Consecutive observations, extending over many weeks, on the hemoglobin percentage and the bilirubin output (see Text-fig. 4; also McMaster, Broun, and Rous[8]) bring out the presence of a relationship between the two which, on cursory inspection, would appear to be undisturbed by normal influences other than exercise. But there are hidden factors which act to distort this relationship. Whenever the hemoglobin percentage is stationary or rising, such blood destruction as may be going on is, of course, wholly concealed through the hema-

[25] Brugsch, T., and Yoshimoto, *Z. exp. Path. u. Therap.*, 1910–11, viii, 639.

topoietic activity; and when the percentage falls gradually, the concealment continues, though now it fails to mask completely the destructive process. Under such circumstances the bilirubin yield cannot be an accurate reflection of the percentage alteration. Furthermore, the amount of it put out in the bile will be directly affected by variations in the degree of pigment conservation. It is remarkable that a daily yield which is the resultant of blood changes that are partially concealed and of a pigment conservation which may vary should so nearly reflect gross alterations in hemoglobin as indicated by the percentage value of blood specimens. If one chooses to think not in terms of this latter substance but in those of the cells in which it is lodged, and ignores intercurrent changes in the color index, then the bilirubin yield becomes a more or less accurate expression of cell mortality. On first view it appears to be a strikingly faithful one. When the cell population is large or small, the number of cell deaths should, other things being equal, likewise be large or small, and so too with the bilirubin output. Such is the actual case. Indeed, the variations in output so closely follow those in the hemoglobin percentage as to give the impression that cell deaths are registered practically at once, that is to say within a few hours, in terms of bile pigment. But always one has to reckon with masked blood destruction and pigment conservation as disturbing factors. Here and there in the charts their influence is manifest. Whenever a noteworthy remission from anemia occurred, the increase in cell number evidenced by the mounting per cent of hemoglobin involved, by corollary, an increase in the eventual number of cell deaths. That this number increased even while the repair was going on may be inferred from the rise in the bilirubin yield. But here a curious fact may be noted. The rise did not parallel but actually preceded that in the hemoglobin per cent. Either, as repair began, more cells were destroyed than ordinarily, or else the need for bilirubin conservation had lessened. As a matter of fact, proof has been brought in a previous paper of a special corpuscular wastage during recovery from anemia,[26] traceable to the circumstance that many of the cells placed in circulation by the hematopoietic tissue are unfit to withstand the exigencies of life there. It will further

[26] Robertson, O. H., and Rous, P., *J. Exp. Med.*, 1917, xxv, 665.

be seen from the charts that whenever a marked drop in the hemoglobin percentage occurred, uninduced by artificial aids, the bilirubin yield fell simultaneously, but generally to a much more marked extent. The factor of replacement must have been of relatively slight importance at such periods, since the marrow was inactive as proven by the small number of circulating reticulocytes. In four specimen instances (see Text-fig. 4; also the curve in a previous paper[8]) the hemoglobin dropped from 118 per cent to 80 per cent, 99 per cent to 70 per cent, 92 per cent to 76 per cent, and 93 per cent to 62 per cent, whereas the bilirubin decreased from 6.2 mg. to 3 mg. (118 per cent to 57 per cent), 4.4 mg. to 2.2 mg. (99 per cent to 46 per cent), 5.3 mg. to 2.2 mg. (92 per cent to 38 per cent), and 5 mg. to 2.9 mg. (93 per cent to 54 per cent) per hour respectively. An increasing conservation of pigment as the anemia gradually developed would explain these findings.

Instances of the sort referred to, in which the drop in the hemoglobin percentage must have come about in the main through a progressive depletion of the current stock of cells without adequate replacement, and in which the bilirubin output decreased concurrently as a consequence of the lessening of cell population with, by corollary, a lessened number of cell deaths from "natural causes," may be contrasted with those others in which a decrease in blood pigment was induced by extraneous influences (Text-figs. 5 and 6). Here the bilirubin yield mounted while the anemia was developing, as would follow from the excessive mortality in a thinning population of corpuscles. It is evident that the changes in the bilirubin output of intubated animals from day to day should aid one to tell whether a given anemia is the expression of ordinary wear and tear on the corpuscles in the lack of the usual cell replacement, or whether, on the other hand, it results from increased blood destruction despite what the hematopoietic tissue may be doing in the way of replacement.

SUMMARY.

In dogs intubated for the collection of all of the bile, a marked falling off in the yield of bilirubin is regularly to be noted after operation, followed soon by an anemia of secondary character. Though, in the absence of complications, the anemia is mild, it persists despite the

excellent general condition of the animal. Intercurrent changes in the hemoglobin percentage take place from time to time, and these are accompanied by very similar fluctuations in the bilirubin quantity. At first inspection the data strongly suggest that blood destruction finds accurate quantitative expression in the yield of bile pigment. But this is not the case. True, the destruction finds expression in terms of bile pigment and practically at once; and the data support the conception that bilirubin has no other sources besides the hemoglobin of destroyed blood. But our experiments show that the amount of it put forth during the development of an anemia from gradual blood destruction, either intercurrent or induced, is far below that derivable from the net quantity of hemoglobin disappearing from the circulation. The discrepancy is referable to a process of pigment conservation which varies in proportion to the body need.

We have shown in a previous paper that bile pigment may on occasion be absorbed from the intestinal tract. This fact and those reported in the present communication lead one to question the accepted view that bilirubin is mere waste material eliminated by way of the liver. The anemia which develops upon total loss of the bile is not improbably consequent on the pigment loss therewith.

Current methods of computing the rate of normal and pathological blood destruction from the bilirubin (or urobilin) yield are unsound in principle and open to large error in practise. Yet there is no doubt that day-to-day variations in the output of bile pigment result from changes in the blood; and on occasion they provide enlightening evidence of the nature of hemic events.

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STUDIES BASED ON A MALIGNANT TUMOR OF THE RABBIT.

III. INTRATESTICULAR TRANSPLANTATION AND CLINICAL COURSE OF THE DISEASE.

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When a satisfactory method had been devised for the transplantation of the tumor which forms the subject of this series of papers (1), a systematic study of the tumor was undertaken, using the method of correlation of clinical and pathological phenomena. The immediate object of these investigations was to obtain a general conception of the behavior of the tumor as an agency for the production of disease, with a view to utilizing this knowledge as a basis for future work. It had been found (1) that under favorable conditions, the tumor was capable of producing a very maligant disease, while under other conditions, it was difficult to obtain even a primary growth. It appeared, therefore, that by the use of a variety of methods of transplantation, one might obtain results which would permit an estimation of the relative importance of the several groups of factors which were concerned in determining the results of transplantation and would give some insight into their mode of operation.

These investigations necessitated a long series of experiments which extended over a period of nearly 2 years. During this time, the tumor was passed through more than twenty generations; inoculations were made into the testicles, the skin and subcutaneous tissues, the muscles, the anterior chamber of the eye, the brain, the peritoneal cavity, and the blood stream. All these animals were studied clinically and pathologically as individual subjects of disease. Thus far, only male rabbits have been used; they have been carefully selected and have been kept in individual cages, and given the best care and attention. With this material, we have been able to study phe-

nomena of tumor growth and of animal resistance, and to correlate these with clinical and pathological effects under favorable and unfavorable conditions of transplantation.

In reporting the results of these investigations, it seems advisable to begin with a description of the growth obtained by various methods of transplantation and the clinical effects observed in tumor-bearing animals, in order to provide a general perspective of the disease to which individual phases of the subject may be related. The present paper will deal, therefore, with the results obtained by testicular inoculation, while the results of other methods will be presented in the next paper of the series (2). In view of the complexity of the conditions which influence the results of transplantation in one way or another, statistical data will be omitted as far as possible until such time as they can be introduced and analyzed to advantage. At present, any attempt to deal with the subject from this standpoint would only lead to confusion unless accompanied by a great deal of explanatory material which would carry us beyond the limits which have been assigned to these papers.

Intratesticular Transplantation.

Methods and Material.

The description of the results obtained by intratesticular transplantation is based upon the study of a large series of rabbits. These animals were inoculated with tissue fragments or with a cell emulsion prepared according to the method described in a previous paper (1). With few exceptions, they were inoculated in only one testicle, which was usually the right, so that there was only one primary focus of tumor growth.

Results.

Percentage of Tumor Growths.—Intratesticular inoculation of finely minced tumor tissue or of a homogeneous cell emulsion prepared from actively growing tumors yielded a definite growth in nearly all animals. Occasionally, the growth was slight, and, in rare instances, no growth could be recognized by ordinary methods of clinical examination, but the percentage of positive results was rarely less than 90 and as a rule was 100 per cent.

Incubation.—Inoculation was usually followed by a slight diffuse swelling of the testicle which disappeared within 24 to 48 hours, leaving a small area of thickening or induration at the point where the tissue was discharged. This in turn diminished or disappeared within 3 to 5 days and was followed immediately by the development of one or more circumscribed nodules or by a diffuse enlargement of the testicle with ill defined areas of thickening. These changes marked the appearance of a definite tumor growth. During the earlier generations, this period varied from 5 to 10 days, but with later generations, the time has been appreciably shortened and now rarely exceeds 5 days.

Growth Characteristics.—The growth of the tumor was usually periodic in character. As far as could be determined by palpation, there was a steady increase in size for a period of 2 to 3 weeks before any interruption occurred. By this time, the tumors in the majority of animals had reached a size of from 1 to 3 cm. in diameter. Occasionally, complete regression occurred after this first cycle of growth. This was especially true of slowly growing tumors in young or adult animals.

In a second group of animals with unusually active tumor growths, there was apt to be a sudden development of edema in the scrotum and adjacent tissues, followed by rapid regression, the tumor softening and diminishing in size. This phenomenon of crisis, as it may be termed, was frequently followed by complete resolution of the primary tumor, but many of these animals subsequently developed secondary growths. In other instances, the primary tumor was only temporarily affected.

The course of events in a third and comparatively large group of animals differed from that of the second in that the edema was less marked, and while growth was arrested, there was comparatively little regression, or even though there was an extensive edema, little or no regression took place.

Finally, there was a fourth group of animals in which growth of the primary tumor was active or of moderate degree but progressed with only slight or brief interruption at this period.

Small or feebly growing tumors, which formed only a small proportion of the whole, not infrequently began to regress at about the end

of the 2nd week, but usually the first critical period in the growth of primary tumors occurred at or near the end of the 3rd week after inoculation and was remarkably uniform in this respect. This was not peculiar to testicular tumors but was observed after all forms of inoculation and is worthy of especial emphasis as a feature of the behavior of the transplanted tumor.

The interruption of growth which occurred at this time was usually of short duration and rarely extended beyond the 4th week. Growth was then renewed with greater or less activity. In many instances, the primary tumor increased actively, involving the entire testicle and extending up the cord into the abdominal cavity in the form of a continuous mass or as discrete nodules which might be few in number and comparatively large, or small and very numerous. It sometimes happened that the testicle was drawn into the abdominal cavity and growth continued in this location. In other instances, a testicular mass developed which involved tunics and scrotum and not infrequently reached a size of 5 to 7 cm. in length by 2 to 3 cm. in diameter.[1]

In a smaller proportion of the animals, the growth which occurred after the interruption of the 3rd or 4th week was less rapid than before or was more circumscribed in character. In some instances, the primary tumor remained comparatively small (1 to 3 cm. in diameter), while in others, it reached a very large size but was confined within the scrotum.

After the 4th or 5th week, periodic changes in the activity of primary tumors were less evident. By this time, the animals of a series had become separated into three fairly distinct groups. The first included animals in which growth appeared to have reached its limits, and the tumors were either quiescent or definitely regressing; the second group was composed of animals with slowly but irregularly growing tumors; while the third was composed of animals with actively growing tumors. With few exceptions, however, what took place was still of an intermittent character, resolution being interrupted from time to time by periods of quiescence or even by a slight renewal of growth, while the growth of more active tumors was

[1] See Pearce and Brown (1), Figs. 1 to 4.

likewise interrupted by periods of quiescence or slight regression. The exceptions to this rule occurred with tumors of an unusually malignant character and less often in instances of unusually rapid resolution in which either process took place with no appreciable interruption.

Metastases.—Metastases developed in the majority of animals, and there were few organs or tissues in which secondary growths were not found, but a detailed report of this feature of the transplanted tumor will be reserved for a future communication. At present, it is sufficient to say that extension of the growth through the inguinal canal and the formation of implantation metastases in the abdominal cavity occurred as early as the 3rd week after inoculation, and metastases by lymph or blood vessels to distant parts of the body made their appearance anywhere from the 3rd to the 5th week.

Termination.—The ultimate fate of the tumor or of the tumor-bearing animal was variable and depended upon a number of factors to be discussed in a subsequent paper. As has been intimated, the growth was suppressed very quickly in some instances; while in others, resolution and apparently complete recovery took place after the tumor had grown for several weeks or months, and this sometimes occurred even after extensive metastases had developed. More often, however, the occurrence of metastases was eventually followed by death. In some instances, death was due to mechanical causes, such as intestinal obstruction, or pressure on the spinal cord, or to mechanical interference with nutrition as in the case of metastases affecting the jaws and especially the region of the lower incisors, but a considerable proportion of the animals showed none of these complications.

Finally, there was a third group of animals in which a condition of equilibrium became established, and for months there was no decided change in one direction or the other, leaving the outcome uncertain even after prolonged observation.

The probable mortality in different series of animals cannot be estimated with accuracy owing to the fact that few series were held intact for more than 2 to 3 months and that it is impracticable to prolong the period of observation sufficiently to obtain a definite end-result. In general, it may be said, however, that within a period of 2 to 3 months, the mortality varied from nil to 100 per cent, depending

upon a number of factors, such as the composition of the series as regards the age and breed of the animals used, the state of the inoculating material, and especially the season at which the experiments were carried out. Under ordinary conditions, from 20 to 30 per cent of the animals apparently recovered during an observation period of 2 to 3 months, while 20 to 40 per cent died, leaving from 40 to 60 per cent in which the outcome was not determined. These proportions are significant as indicating a resistance distribution which is relatively constant for different groups of rabbits. That is, in any group of five rabbits, there are usually one of relatively high resistance, one of distinctly low resistance, and three which are intermediate and comparable to one another.

Duration.—For the same reasons as those given above, the duration of tumor growth or the length of survival of tumor-bearing animals can be stated only in general terms. The growth of the tumor in surviving animals varied between 10 days to 2 weeks and an indefinite period of more than 6 months. Few animals showed complete suppression of tumor growth before the end of the 2nd or 3rd week. In most instances, final regression began between 6 weeks and 2 months after inoculation. That is to say, the turning point was reached at about this time, and one was able to forecast the probable outcome with a considerable degree of accuracy. Resolution and absorption required a longer time, and in the case of large tumors were rarely complete before the end of the 3rd month, and frequently the time was much longer.

Death from the tumor usually occurred between 7 and 12 weeks after inoculation, the period of greatest frequency being from 7 to 10 weeks. Occasionally, the course of the disease was more prolonged, and the animal survived 4 to 6 months after inoculation. More often, the disease was fulminating in character, and death occurred within 6 to 7 weeks.

Relation between Growth and Malignancy.—In concluding the section dealing with phenomena of tumor growth, it should be made clear that no constant relation was observed between the rate of growth or size of primary tumors and the malignancy of the tumor as indicated by the occurrence of metastases or by the course of the disease. In fact, it appeared that the relationship was more often a

reverse than a direct one. That is, tumors which grew most actively during the first 2 to 3 weeks were usually suppressed and rarely gave rise to extensive metastases or caused the death of the animal. In other instances, large primary tumors developed which were confined within the tunics and the scrotum and produced no serious consequences. The most malignant tumors, on the other hand, were characterized by a moderate but steady rate of growth with invasion of the tunics and extension up the cord into the abdominal cavity. Similar conditions obtained with reference to metastatic tumors. The most distinctive feature of the two classes of tumors was, therefore, the manner in which the animal responded to the presence of the tumor rather than the rate or size of the growth.

Immunity.—The subject of immunity of tumor animals was not investigated extensively, but it may be said that, with rare exceptions, animals that had once been inoculated with negative results were insusceptible to a second inoculation. In like manner, animals that had apparently recovered could not be reinoculated successfully by any of the usual routes. The duration of this resistance is not known, but reinoculations made as late as 6 months after recovery still gave negative results.

Symptomatology of Tumor Animals.

In describing the symptomatic effects produced by this tumor, we shall confine ourselves almost entirely to such conditions as can be detected by ordinary methods of observation supplemented by records of weight.

The first recognizable change which occurred in tumor animals was of a local character and consisted of an enlargement of the regional lymph nodes which appeared almost immediately after inoculation and gradually increased for upwards of 7 to 10 days. This reaction in the regional lymphatics was usually followed by similar changes in other groups of nodes. As a rule, the initial swelling diminished towards the end of the 2nd week, leaving the nodes still somewhat enlarged and distinctly indurated. This condition persisted with but little change until the turning point in the disease was reached. At that time, the nodes showed either a further increase in size or underwent a decided atrophy. Thus, if the growth was progressive,

further enlargement of the lymph nodes usually occurred, but when resolution took place, the nodes diminished in size and in some instances became extremely small and indurated. In this respect, there was a striking contrast between the conditions presented by animals in which the disease pursued a rapidly fatal course and those which survived for a longer time. In acutely fatal cases, the lymph nodes were usually smaller than normal, whereas in the more chronic cases, they tended to be greatly enlarged and were succulent or edematous in character. During the late stages of the disease, therefore, either a hypertrophy or atrophy of the superficial lymph nodes might occur, depending upon the course of the disease.

During the first few weeks after inoculation, there were no decided changes in the general physical condition of animals with actively growing tumors. They usually gained in weight, and at times, the increase was somewhat greater than that of normal animals under similar conditions, and was usually greater than that of animals in which the growth of the tumor was slight or of short duration. In fact, these animals not infrequently showed an actual loss in weight.

The first evidence of a nutritional disturbance appeared as an accompaniment of the phenomenon of suppression of tumor growth and consisted of a cessation of the normal increase in weight or a sudden loss in weight which in extreme cases amounted to several hundred grams. This condition might be transient or might last for from 10 days to 2 weeks, and was frequently associated with some change in disposition. In the milder cases, this usually took the form of a nervous excitation, while in other instances, there was a decided apathy with dryness of the skin, roughening of the coat, and loss of appetite which doubtless accounted for some part of the loss in weight and the prolongation of this condition.

These changes in physical condition appeared to be influenced largely by the extent of the tumor growth and by the suddenness with which the reversal of this process took place. With slight or transient growths, there might be no appreciable alteration in the physical condition of the animal. If, on the other hand, the growth had reached a considerable size and regression began abruptly, the change was usually marked. In some animals with large primary tumors or with metastases to internal organs, in which the change

from progression to regression took place slowly, the loss in weight might be barely perceptible, while the change in disposition was very decided. In some instances, there was no more than an apathy, while in others, there was a marked nervousness which was frequently accompanied by a pronounced exophthalmos. This latter condition was also noted in other classes of animals. It was frequently transient, but at times persisted for weeks, or disappeared and recurred from time to time with successive periods of progression and regression of the tumor. It was not permanent, however, but disappeared as the tumor was absorbed.

As absorption of the tumor progressed, the general condition of the animal tended to improve. Animals that had made a prompt recovery showed a decided improvement in their general appearance, with a substantial increase in weight, and remained in excellent physical condition for months. In cases in which recovery was slow and at times uncertain, there was evident impairment of physical condition and the animals were subject to intercurrent infections and their powers of resistance to such infections were diminished as contrasted with those of the first group.

When the growth was not arrested, the disease followed either an acute or chronic course, terminating in death, and the changes observed in the physical condition of the animal varied accordingly. Those animals in which the tumor grew most actively usually remained in excellent condition until shortly before death. If death occurred suddenly as a result of accidental interruption of some essential process, there might be no obvious deterioration in the physical condition of the animal, depending upon the progress which the disease had made at the time of such an occurrence. In the absence of accidental complications, the animal began to lose weight and strength, the conjunctivæ and mucous membranes became pale, movements were sluggish, the skin was relaxed and was thin and delicate, while the coat was dry and roughened, the eyes were sunken, the lids drooped, and towards the end, there was not infrequently an excessive flow of lacrimal and mucous secretions.

The pulse rate of these animals was so variable that no estimation of its character can be given. The temperature during the early periods of tumor growth varied very little from the normal, but as the

disease progressed, there was an apparent tendency to an elevation of temperature which was relatively slight in most animals. Occasionally, however, the temperature was subnormal, and a decided elevation followed by a fall below normal immediately preceding death was noted in several instances.

The conditions described made their appearance anywhere from a few days to 2 or 3 weeks before death and increased with greater or less rapidity according to the progress of the disease. It appeared that these symptoms were rarely if ever the result of tumor growth *per se*. In fulminating cases of malignancy, metastases were nearly always present in one or both suprarenals, and they were frequently found in the pituitary body, hence it is difficult to determine the part played by tumor growth and that which may be ascribed to accidental involvement of organs such as these.

In this connection, mention should be made of the occurrence of clinical conditions simulating Addison's disease—minus the element of pigmentation—and of genital atrophy from metastases in the suprarenals and pituitary body respectively, and the occasional occurrence of a peculiar skeletal overgrowth for which as yet no cause can be ascribed. The conditions which suggested the Addison syndrome occurred in their most typical form in animals which did not show extensive tumor metastases, the presence of metastases in the suprarenals being indicated by an extreme weakness and pallor of mucous membranes which were out of proportion to other evidences of tumor involvement. The picture was so characteristic as to lead one to believe that a close approximation of the human disease might be produced experimentally by inoculations made directly into the suprarenal glands.

In like manner, the presence of a metastasis in the pituitary body was diagnosed on several occasions by the occurrence of an extreme atrophy of the uninoculated testicle which under ordinary circumstances retained its normal size and condition or underwent an hypertrophy. The other element of the Fröhlich syndrome (adiposity) was rarely seen in these animals, due to the presence of metastases in other organs or to the acute course of the disease, but in one instance, the complete picture of marked accumulation of fat and extreme atrophy of the testicle occurred in typical form.

The picture presented by animals in which the disease pursued a chronic course extending over some 4 to 6 months was quite different from that of the acutely fatal affection. The change from a condition of well-being to one of extreme cachexia occurred insidiously, and at the end, the picture was complicated by the presence of some intercurrent infection. There was a gradual loss of weight and of strength; the animal became apathetic and the movements were sluggish; the hair was dry and roughened and the eyes sunken as in the preceding case, but the skin of these animals was also dry and thick, or of a parchment-like texture, and was covered by fine scales, and frequently there was a decided thinning out of the hair. In addition, areas of mucoid infiltration were frequently present in the skin and subcutaneous tissues of the genital region, and as the end approached, they occasionally appeared elsewhere. These animals maintained a firm hold upon life in the presence of extensive tumor metastases, and death was not infrequently due to some form of bacterial infection.

It is obvious that conditions other than those described were present in all classes of tumor-bearing animals, but as yet there are many aspects of the disease which we have not been able to investigate in a systematic way. The blood, for example, showed distinct changes, the best known features of which were an anemia, a mononucleosis, and some change in coagulability. Under certain circumstances, the coagulation time was diminished, but in cases of high malignancy, it was apt to be increased and instances were noted in which the blood either failed to coagulate after prolonged standing or formed a very imperfect clot.

CONCLUSION.

Since the paper which follows this will deal with the results obtained by other methods of inoculation, the discussion of the transplanted tumor and its effects upon the animal organism may be conveniently deferred until the report dealing with these subjects has been completed. From what has been said, it is apparent, however, that when transplanted by the method described, the tumor under consideration is capable of producing a highly malignant disease. But even under these conditions, the results of transplantation are

extremely variable, which indicates that the factor of animal resistance is of equal if not greater importance than that of the capacity for growth possessed by the tumor cells.

SUMMARY.

The results obtained by intratesticular inoculation of a malignant tumor of the rabbit are presented in the form of a general summary covering the phenomena of growth and spontaneous regression of the tumor and the clinical evidence of disease in tumor animals.

Under ordinary circumstances, growth is obtained by this method of inoculation in practically all animals, and the majority of them show metastases.

The ultimate fate of tumor animals is variable. Spontaneous regression is of frequent occurrence, and apparent recovery may take place even after extensive metastases have developed. More often, the disease terminates fatally. The course of the disease may be fulminating in character with death occurring 6 to 7 weeks after inoculation, or it may be prolonged over a period of more than 6 months, but death usually occurs between 7 weeks and 3 months after inoculation.

The symptomatology varies with the course of tumor growth but is in general that of a debilitating disease of an acute or chronic character in which the picture is frequently complicated by symptoms referable to mechanical causes or to one or more of the glands of internal secretion. The most prominent of these are due to the presence of metastases in the suprarenals or pituitary body, in which case symptoms of Addison's disease or diseases of the pituitary may be superimposed upon those attributable to the presence of extensive tumor growths. In the absence of such growths, however, the symptoms of suprarenal or pituitary involvement may dominate the picture.

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STUDIES BASED ON A MALIGNANT TUMOR OF THE RABBIT.

IV. THE RESULTS OF MISCELLANEOUS METHODS OF TRANSPLANTATION, WITH A DISCUSSION OF FACTORS INFLUENCING TRANSPLANTATION IN GENERAL.

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PLATES 44 AND 45.

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As stated in previous papers of this series (1, 2), rabbits inoculated in the testicles with tissue fragments or a cell emulsion of the tumor under consideration almost invariably develop a local growth. These tumors frequently metastasize and give rise to physical signs and symptoms of disease which differ somewhat according to the course of the disease and the location of metastases in individual animals. Under favorable conditions, the progress of the growth is rapid, and while some animals apparently recover, many of them die either acutely (6 to 8 weeks), or after the lapse of several months, presenting a picture not unlike that of malignant disease in man.

In reporting the results of intratesticular inoculation, emphasis was placed upon the phenomena of disease or the clinical effects of the tumor and the reaction of tumor-bearing animals rather than upon the immediate results of inoculation. When other methods of transplantation are employed, however, conditions are altered to such an extent that the results must be presented from a different point of view. The percentage of takes, the size attained by primary tumors, the incidence of metastases, and the duration of the growth become the standards of measurement as in most experimental work with the tumors of laboratory animals.

Thus far, the efforts which have been made to propagate the tumor may be divided into three classes: (1) inoculation of uninjured tumor

fragments or tumor cells; (2) inoculation of cells which have been injured, as by repeated freezing and thawing; and (3) inoculation of desiccated tumor tissue and of Berkefeld filtrates. A few attempts have also been made to transmit the growth to other species of animals. The results of experiments other than intratesticular inoculation of uninjured tumor cells will be reported in the order enumerated above.

Transplantation of Uninjured Tumor Cells.

In addition to the intratesticular route, seven methods of transplantation have been studied, including subcutaneous, intracutaneous, intramuscular, intraocular, intracerebral, intraperitoneal, and intravenous inoculations. For convenience, the results obtained by these methods may be considered collectively.

Methods and Material.

The selection and preparation of the material used for all inoculations in this group were essentially the same as those described in the second paper of the series (1) and need not be repeated. Both tissue fragments and cell emulsions have been used for subcutaneous and intracutaneous inoculations, but cell emulsions were used for all other inoculations. Injections were made with a syringe and needle (Fournier tuberculin syringe, graduated in 0.01 cc.).

Subcutaneous inoculations were made in the scrotum, the groin, and the axilla or pectoral region, intracutaneous inoculations on the ventral surface of the sheath, and intramuscular inoculations in the muscles of the inner and posterior aspect of the thigh.

Intraocular injections were carried out under a local anesthetic. The cornea of one eye was punctured at its upper and outer margin with a corneal knife; a small amount of the aqueous humor was permitted to escape and the emulsion introduced into the anterior chamber.

Intracerebral inoculations were done under a general anesthetic. A small incision was made in the skin over the middle of the right frontal bone near its anterior extremity. With a trephine, a small opening was then made in the bone through which the needle was introduced and the injection made into the anterior portion of the

right frontal lobe of the brain. These procedures were carried out aseptically.

The intravenous inoculations were made through an ear vein and the intraperitoneal by injection into the peritoneal cavity.

For the sake of uniformity, male rabbits were used in all cases, and with the exception of one group of experiments, in which combined intratesticular and subcutaneous or intracutaneous inoculations were studied, animals were inoculated at only one point.

In most instances, a single series of five or six animals appeared to be sufficient to indicate the results which might be expected from the use of a given method and the probable utility of the method for experimental purposes, but with the intracutaneous and subcutaneous routes, several series of animals were studied.

Results.

Tumor Incidence.—Inoculations made into the brain and the anterior chamber of the eye gave positive results in all but one animal. In this instance, the emulsion was apparently washed out of the eye and a second inoculation was successful. The percentage of takes from intramuscular and intracutaneous inoculations was also high (80 to 90 per cent), but with the subcutaneous route, it was often difficult or impossible to determine whether there was a definite growth or not on account of the marked local reaction. A small nodule developed at the site of inoculation in all animals, but in most instances, this nodule was composed largely of granulation tissue. From a study of these lesions, it appeared that the cells introduced survived for several days and that there was probably some growth but it was only in an occasional animal that a clearly defined tumor developed.

If for present purposes, therefore, such indefinite results be regarded as negative, the incidence of tumors from subcutaneous inoculations may be placed at from 20 to 25 per cent, or one animal out of four or five. For example, in a series of sixteen rabbits inoculated in the subcutaneous tissues of the groin, four developed fairly large, actively growing tumors, and in another series of fourteen animals, three developed tumors. Only once was this proportion exceeded.

In this instance, three out of five animals gave positive results, but there was still only one animal with a good tumor growth comparable to those regarded as positive in other series.

Attention may be called to the fact that this ratio of one in five corresponds roughly with the proportion of fulminating cases of malignancy which occurred after intratesticular inoculation and that it is also significant in that it corresponds with an incidence of low resistance displayed by rabbits to a variety of unrelated experimental conditions. It has been given practical application in this laboratory for a number of years in planning and evaluating toxicologic experiments and experiments dealing with biological aspects of infections such as syphilis. Since no normal rabbit has died thus far from tumor inoculations in the subcutaneous tissues, this method of inoculation has been utilized in the study of crossed relationships of resistance to tumor and to syphilis as one means of identifying animals with the lowest tumor resistance. The reactions to intracutaneous and intramuscular inoculations have also been helpful in this respect as a means of determining intermediate degrees of resistance.

Intraperitoneal and intravenous inoculations, as determined by autopsy, gave practically the same results as subcutaneous inoculation. Each of these series consisted of five rabbits. One animal of each group was killed at the end of 3 weeks; one of the intravenous animals became very weak and was killed after 4 weeks, while the others were killed at from 6 to 8 weeks after inoculation. The results of the two series were identical. In each, there were three animals with no evidence of active or healed tumors, one animal with what appeared to be small tumor nodules which had healed, and one with active multiple growths. Both of these were diagnosed during life, in one case by the development of weakness and anemia suggestive of Addison's disease (intravenous series) and in the other by a palpable growth in the abdominal wall.

Incubation.—The incubation period which might be given for tumors produced by different methods of inoculation would naturally vary according to the opportunity for detecting the presence of a growth. Thus, with the intravenous and intracerebral inoculations, the first evidence of a growth was symptomatic, and in most instances,

these symptoms did not appear until nearly 3 weeks after inoculation. With inoculations made into the anterior chamber of the eye on the other hand, a visible growth could be detected within 72 hours. In other instances, the first signs of growth were derived from palpation, and the results varied somewhat according to the extent of the reaction aroused in the tissues at the site of inoculation.

However, a growth in the skin or muscles was usually recognizable within 5 to 7 days after inoculation, and within 7 to 14 days after inoculations in the subcutaneous tissues.

Growth and Termination.—The character and extent of the growth obtained in these experiments differed according to the method used. It was found that during the first few weeks the growth of the tumor could be followed to excellent advantage in the anterior chamber of the eye, which served the purpose of a culture medium in which the proliferation of tumor cells and the growth of nutrient vessels could be observed directly. In this location, the growth first assumed the form of an extremely delicate membrane or of an intricate network of fine thread-like processes of a pale opalescent appearance which floated freely in the aqueous humor, except for an attachment at their point of origin. The growth of these structures was accompanied by the development of an equally delicate network of vessels which originated from subjacent parts of the iris. These vessels were so delicate that they were apt to be ruptured by any unusual excitement of the animal, and hence were the source of rather frequent and profuse hemorrhage.

For the first 2 or 3 weeks, the growth of these tumors was very rapid, and by the end of the 3rd week, the anterior chamber was usually filled with an irregular lobulated mass of an opaque white or opalescent appearance mottled with areas of necrosis and hemorrhage. When this point had been reached, growth became less active on account of the confining influence of the tunics of the eye. Eventually, however, the bulb became deformed and tended to protrude, and in some animals, the growth penetrated to the outer surface of the eye. The course of events was not followed beyond this point, the experiments being terminated on account of the tendency to hemorrhage and secondary infection of the exposed tumor mass.

In the group of animals studied, there was only one which showed signs of permanent regression of the ocular tumor during a period of 3 to 6 weeks observation. The tumor in this instance developed from the corneal incision and was located chiefly in the conjunctival tissues. On the other hand, no metastases were demonstrated in any of these animals.

Tumors in the brain grew very rapidly, exhibiting a tendency to spread rather diffusely along the surfaces of the meninges and over the walls of the ventricles, and in most instances, there was an abundant growth about the choroid plexus. Of the five animals comprising this group, one died 12 days after inoculation and another on the 16th day as a result of a growth in the cranial cavity. The three remaining animals showed pronounced symptoms of intracerebral pressure and were killed on the 17th day. None of these animals showed visible metastases, and it was apparent from this experiment that rabbits inoculated in the brain would rarely survive longer than 3 weeks.

The growth of tumors in the muscles was also rapid. Irregular lobulated masses or multiple nodules developed at the site of inoculation, and within 2 to 3 weeks, some of these reached a size of from 1 to 3 cm. in diameter. They invaded the muscle and extended centrally for a considerable distance, but in the small group of animals studied thus far, all the primary tumors have undergone spontaneous regression, and with one possible exception, no distant metastases have been found.[1]

The tumors produced by intracutaneous inoculation were usually small and rarely exceeded 1 cm. in diameter. They were smooth and elastic, and at the height of their activity, not infrequently invaded the surrounding skin and showed surface ulceration. The fate of these tumors was variable. In animals inoculated on the sheath only, the tumors persisted for a comparatively short time and no metastases were observed, but the skin tumors of a few animals

[1] The animal of this series which showed the most persistent growth was subsequently inoculated with *Treponema pallidum* and developed a severe generalized syphilis. At the height of this disease, the animal was given a second tumor inoculation in the subcutaneous tissues. There was a slight local growth, and when the animal was killed 6 weeks later (6 months after the first inoculation), an actively growing metastasis was found in one of the adrenals.

inoculated in both the testicles and the sheath continued to grow parallel with those in the testicle and metastases developed in the inguinal lymph nodes which may have been derived from the tumor in the skin.

With subcutaneous inoculations, there was a further reduction in the extent of the primary growth. As has been mentioned, small, intensely indurated or elastic nodules of 3 to 8 mm. in diameter developed at the site of inoculation in all animals, but by the 10th day, they usually began to soften and diminish in size, leaving only a small fibrous mass. Clinically, such cases may be regarded as negative in that the growth of tumor cells could not be distinguished from the granulation tissue which formed the greater part of these lesions.

In other instances, the growth assumed the form of an irregular elastic nodule or of a firm plaque which continued to increase in size for 2 to 3 weeks and was almost invariably associated with a marked enlargement of the regional lymph nodes as shown in Figs. 1 and 2. This feature of the reaction was at times helpful in distinguishing between small tumor nodules and masses of granulation tissue. In both cases, the regional lymph nodes became enlarged, but the enlargement was much greater when there was a definite growth of tumor cells. This condition is illustrated in Figs. 3 and 4 which are taken from two animals of the same series which were inoculated in the mammary line on the right side of the thorax. In Fig. 4, the nodule at the site of inoculation is barely perceptible and the enlargement of the adjacent lymph nodes is much less than in Fig. 3 which shows a small but definite tumor nodule.

This was the picture usually presented, and in cases of small nodules of uncertain character, the associated lymphoid reaction was helpful in arriving at a diagnosis of the probable extent of the tumor growth which had taken place. For the same reasons, inoculations in the mammary line were preferable to those made in the axilla or groin.

Subcutaneous tumors were either single or multiple; they were usually lobulated and rarely exceeded a centimeter in diameter. As a rule, regression began within 2 to 3 weeks after inoculation, but in exceptional instances, growth was more active and more prolonged and the tumors attained a size of 2 or more cm. in diameter. An

example of a growth of this type is given in Figs. 5 to 8 which represent stages in the development of a tumor from 9 to 37 days after inoculation. An interesting feature of the condition here shown is the reduction in the size of the inguinal lymph nodes which took place during the later stages of tumor growth or preceding any appreciable degree of regression.

Thus far, no metastases have been found from primary tumors in the subcutaneous tissues of normal rabbits; all of them have undergone spontaneous resolution, and 4 to 5 weeks was the extreme limit of their activity. This fact should be emphasized, since in certain classes of syphilitic rabbits, inoculation by this method will lead to the development of metastases and to the death of the animal.

Little can be said of the growth which takes place after intraperitoneal or intravenous inoculation. In the two instances of this kind in our series, the growth was generalized, and one animal (intravenous) would undoubtedly have died, but the probable fate of the other is uncertain.

In general, primary tumors of the eye, the muscles, the skin, and the subcutaneous tissues showed periods of increased and decreased activity analogous to those described in tumors of the testicles, and in the case of the most active growths, resolution not infrequently began abruptly and at times was preceded by an acute swelling or edematous infiltration of the surrounding tissues.

In spite of the frequency of negative results and the relatively benign character of the growth obtained by intramuscular, intracutaneous, and subcutaneous inoculations, animals inoculated by these routes developed an immunity which was sufficient to protect them from subsequent inoculation by the same route or from inoculations made into the testicles.

The outstanding features of this group of experiments were the varying incidence of primary tumors by different methods of inoculation, the relatively benign character of the growth in the muscles, the skin, and the subcutaneous tissues and the constant occurrence of spontaneous resolution, the absence of metastases, the persistence of the growth in the eye, and the rapidly fatal course of tumors in the brain.

Inoculations with Cells Repeatedly Frozen and Thawed.

It has been observed that comminution of tumor cells, as by grinding, may reduce their activity, and that while the cells survive outside of the animal body for long periods of time at low temperatures, the success of transplantation is usually diminished by repeated freezing and thawing.

In our earlier experiments, it was found that inoculations made with a cell emulsion prepared by grinding the tissue in a mortar gave results which were fully as good as those obtained by the use of small pieces of tumor tissue. This suggested an unusual degree of resistance to mechanical influences, and in order to test this condition further, a series of experiments was carried out with a cell emulsion which had been subjected to repeated freezing, thawing, and grinding.

Method.

The method used was as follows: A cell emulsion was prepared as for ordinary inoculation and divided into two parts, one of which was placed in the ice box for the inoculation of control animals. The other portion was placed in a flask and reduced to a temperature of –20° to –25°C. and kept at this temperature for 30 to 40 minutes. The emulsion was then thawed rapidly by immersion of the flask in warm water and the process repeated. Finally, the frozen material was placed in a mortar and ground before inoculation, and a dark-field examination of the emulsion was made to determine the extent to which the cells had been broken up. Inoculations were made intratesticularly, as usual, and controlled by the inoculation of a second series of animals with the portion of the original cell emulsion which had been kept in the ice box.

Two experiments of this kind were done. In the first experiment, the emulsion was frozen twice before being inoculated into five rabbits. One of these was killed 8 days after inoculation in order to determine microscopically whether there was any evidence of a growth. The four other animals developed tumors after an incubation period of between 2 and 3 weeks as contrasted with 1 week in the controls.

In the second experiment, the emulsion was frozen and thawed five times, but the results were essentially the same as in the first. The incubation period was slightly longer, but all the animals developed tumors.

These experiments show a remarkable resistance on the part of the agent responsible for the transmission of the tumor, but may be explained upon the basis of a failure to destroy the tumor cells, since dark-field examination of the emulsion used in the second experiment showed that while the majority of the cells were fragmented, there were a few that presented an apparently normal appearance.

Inoculations with Desiccated Tumor Tissue and Cell-Free Filtrates.

With a view to determining whether the tumor could be propagated by inoculation of material which contained no living tumor cells, three series of experiments were carried out with desiccated tumor tissue and with cell-free filtrates.

Methods.

Desiccation of the tumor tissue was accomplished by spreading a thin layer of tumor pulp over the bottom of a large Petri dish, freezing, and drying in a vacuum over phosphorus pentoxide while in a frozen condition. An emulsion was prepared from the powder obtained and injected as usual.

The filtrates were prepared from an ordinary cell emulsion, diluted slightly more than usual, by passing through fine, medium, or coarse Berkefeld filters.

Inoculations were made in the testicles, the anterior chamber of the eye, and the brain. The activity of the growth used for the preparations of these materials was controlled in each case by intratesticular inoculation carried out with the fresh cell emulsion.

All these experiments gave negative results. While this does not prove that living tumor cells are essential to the propagation of the tumor, the experiments do show that the causative agent was not capable of passing even a coarse Berkefeld filter or of surviving the process of desiccation as was the case with the chicken tumors studied

by Rous and his collaborators (3). The possibility of the existence of an agent distinct from the tumor cell has not been investigated beyond this point.

Inoculation of Foreign Species.

A few attempts were made to transmit the growth to rats and to guinea pigs by intratesticular and intracerebral inoculation but with negative results in all cases.

DISCUSSION AND CONCLUSIONS.

Taken as a whole, the most striking feature of the behavior of the transplanted tumor, as recorded in this and the preceding paper (2), is the wide difference in the results which may be obtained by varying the method of inoculation. The fact that, under one set of conditions, the tumor is practically incapable of growth, while, under another, it is capable of producing a most malignant disease, and, under still other conditions, produces a growth of considerable proportions but of relatively benign character, affords an exceptional opportunity for the study of phenomena of tumor growth and of conditions which contribute to malignancy or to resistance on the part of the host. It is not our intention, however, to discuss these subjects at this time further than to point out certain features of the experiments reported which indicate the sphere of action of several groups of factors which determine the results of transplantation.

It is obvious that the tumor possesses a capacity for growth which is not exhibited by the cells of normal tissues, and that, under favorable conditions, it is capable of producing a disease which is analogous in many respects to human cancer. It is apparent, however, that neither the extent of the growth nor its effects upon the animal organism are determined solely by the activity of the tumor cells, but that there are a number of factors which contribute to these results, all of which are effective either by direct action upon the tumor cells or through the intervention of the host. In brief, there are three elements which affect all experimental work of this kind. These are the material used, the method, and the animal. The first two of these factors can be maintained at a fair level of constancy

or varied almost at will provided the investigator is sufficiently familiar with his material. Still, the capacity for growth of a given material can be estimated only within certain limits, and at times results will be obtained which can be explained only upon the assumption of a capacity for growth which was much greater or much less than had been supposed. The third factor, however, is the most uncertain of the group. Its value is difficult to estimate and is subject to change, hence an intimate knowledge of the animal is the most important element in the experimental equation. In the experiments reported, there are numerous examples of the influence of each of these factors in determining the results of tumor inoculation.

The capacity for growth possessed by the cells of this tumor is brought out to greatest advantage by inoculations made into the brain, the eye, and the testicle, where large and rapidly growing tumors are produced in almost every animal. When given the advantage of such locations as the brain, the testicle, the anterior chamber of the eye, the muscles, or even the skin of the sheath, growth of the tumor cells stands out in practically all animals as an aggressive and dominant force for a period of from 2 to 4 weeks. During this time, only the exceptional animal appears to be capable of opposing an effectual resistance.

In the subcutaneous tissues, conditions are reversed. Native resistance is sufficient to dominate the situation completely. The tumor grows very little if at all in most animals, and it is only the one animal in four or five whose resistance is so feeble that the tumor is able to reach any considerable size. Even in these cases, however, growth is arrested before metastases develop, and no serious consequences are produced. Obviously, similar results might be brought about by nutritional deficiencies as in the case of ill nourished, old, or diseased animals, but in the present instance, the contingency of a passive nutritional deficiency can be eliminated on account of the ease with which metastases grow in both the skin and subcutaneous tissues of animals whose resistance has been broken. Given this advantage of location, therefore, animals with the least resistance are able to confine the activity of the tumor cells to essentially the same plane as that reached in the most resistant animals inoculated

in the testicles or far below that of intracerebral inoculations. In other words, by merely varying the location of the tumor graft, extremes of resistance may be equalized or made to overlap. Apparently these differences in results are due to the opportunity afforded for bringing the forces of resistance into operation or are matters of advantage and disadvantage.

With the growth of tumors in any location, the resistance of all animals tends to increase, and apparently the extent of the increase varies according to the demand, within the capacity of the individual animal, and, except in the case of tumors in the brain, a point is usually reached at which resistance becomes dominant. In the brain, the extent of the local growth almost inevitably causes death before there is any opportunity to check its progress (2 to 3 weeks). With other forms of inoculation, there is either a temporary arrest or complete suppression of growth, and here again the result is determined in part by the capacity of the individual animal and in part by the advantage given through inoculation. In the case of tumors of the eye, the end-result is unknown, but with tumors in the skin and muscle, complete suppression occurs, and with possible rare exceptions, the growth is controlled before metastases develop.

With testicular tumors, conditions are different. In a single series of ten or twelve animals, one may see every degree of antagonistic activity between the forces of growth and of animal resistance included between the extremes of a slight and transient primary growth and a fulminating case of malignancy. A few animals possess a high threshold resistance which is sufficient to reduce the growth of the tumor to an almost negligible quantity. In a somewhat larger group of animals, the initial resistance is comparatively low, and the tumor grows rapidly, but there is a prompt and efficient reaction on the part of the animal with suppression of growth as by crisis. The opposite of this condition is also seen in animals with slowly growing but rather persistent tumors. Apparently these animals have a relatively high native resistance but are either incapable of a more vigorous reaction or are not aroused to their full capacity. All of these are examples of either a high threshold resistance or a reserve capacity for prompt and vigorous reaction.

At the other end of the scale, there are a few animals with neither native resistance nor reserve capacity sufficient to offer more than a

feeble opposition to the growth of the tumor cells. These animals furnish the fulminating cases of malignancy and occur in a proportion of about one in five in any group of rabbits, or in the same proportion as animals in which the tumor grows actively from inoculations made into the subcutaneous tissues.

Between the two extremes, the opposing forces are more nearly equal, and the balance swings first in one direction and then in the other. A study of the conditions presented by this group of animals is especially instructive. It is obvious that of the two sets of factors concerned in these reactions in a given experiment, the potentialities for growth represented by the tumor are relatively fixed and the source of variation is to be sought in the host.

The renewal of growth in a tumor after a period of inactivity or regression raises the question as to whether this is due to a passive decline in the resistance of the animal which follows a temporary arrest of the growth or is a phenomenon of exhaustion. As has been pointed out elsewhere, periodic changes of this kind are not peculiar to either spontaneous or transplanted tumors but are phenomena characteristic of many diseases, especially those which pursue a chronic course, and in all these conditions the outcome would appear to be determined by the way in which the host responds as well as by the ability to meet the demands which are imposed upon it.

There are many circumstances connected with the growth of this tumor which suggest that the effort put forward by the animal is roughly proportional to the demand. One such instance has been cited in connection with the suppression of rapidly growing and of slowly growing tumors. But apparently this does not hold in all cases. The fact that the animals composing the group in which there is a shifting balance gradually become separated into two subgroups one of which eventually controls the tumor while those of the other succumb, suggests that in one instance the occurrence of periodic changes may be due to a lack of sustained effort or a passive decline in resistance following a temporary suppression of growth, while in the other, there is clearly a failure in the ability of the animal to maintain an effectual opposition to growth which is suggestive of exhaustion.

Going a step further, there is evidence to show that a close relation exists between the local expression of resistance to tumor growth

and the general health of the animal. For example, animals that control their tumors readily show an improvement in physical condition and are not only immune to further inoculation with the tumor but are highly resistant to epizootic infections and to syphilitic infections as well, while constitutional symptoms of disease and feeble resistance to the same class of influences are associated with a defective or feeble resistance to tumor growth.

Moreover, the development of cachexia and the occurrence of death are not necessarily related to either the mass of the tumor or the rate of its growth but to the ability of the animal to confine the growth to a given location. Large primary tumors may produce practically no constitutional symptoms, and animals with large metastatic tumors may remain in excellent condition for months, but with steadily progressive tumors or when showers of metastases appear in the skin, subcutaneous tissues, and other parts of the body at an early period, there is a gradual or rapid decline in physical strength, as the case may be, followed by death, even though the primary tumor may be comparatively small and the total mass of tumor tissue no greater than is frequently found in animals that show no symptoms of disease. In the one instance, the picture presented is that of a slow exhaustion, while in the other, it approaches the condition of a sudden collapse.

In the same way, one finds in any group of apparently healthy and vigorous animals a relatively uniform threshold resistance, while the reserve capacity of these animals varies widely as regards their resistance to tumor growth or to other noxious agencies, and it is the reserve capacity which forms the true measure of their resistance.

In other words, there is a close parallelism between the functioning of those processes which are concerned with the limitation of tumor growth and those which have to do with the maintenance of the general health of the animal. At any rate, each of these processes is conditional upon the other, and both are dependent to some extent upon the same mechanism. In its final analysis, therefore, it is apparent that while the quality of malignancy as brought out in these experiments is conditioned upon the capacity for growth of the tumor cells, the degree of malignancy displayed in any given instance is a function of animal resistance which is not entirely

specific but corresponds closely with the ability of the animal to meet excess demands arising from other causes and, to this extent, is an expression of the functional capacity of a mechanism which is concerned primarily with the maintenance of the general animal economy.

These distinctions as to the sphere of influence of the several groups of factors in determining the results of tumor growth, the manner in which individual animals respond to the presence of the tumor, and the relation of these reactions to the effects of the tumor upon the animal organism are important in that they furnish a basis for an experimental approach to problems of tumor growth and malignancy, and the conceptions which have been outlined above will be developed more fully in future papers dealing with metastases, conditions which influence malignancy, and effects produced by the tumor upon the animal organism.

SUMMARY.

The results of seven methods of transplantation of a malignant tumor of the rabbit are reported and contrasted with results obtained by intratesticular inoculation.

It was found that inoculations made into the brain, the anterior chamber of the eye, and the muscles gave an almost uniform series of takes with the production of comparatively large and vigorously growing tumors. Intracutaneous inoculations were less favorable, while with subcutaneous, intravenous, and intraperitoneal inoculations, a growth was obtained in only 20 to 25 per cent of the animals.

In contrast with the malignant course of the disease produced by testicular transplantation, the tumors produced by methods other than intracerebral inoculation were relatively benign. In all cases, the growth was of a purely local character; in some instances, there was invasion of the surrounding tissues with local or regional extension of the growth, but no metastases developed in distant organs. Tumors in the brain soon caused death as a result of pressure, and the ultimate fate of tumors in the eye was not determined, but the growth produced by other methods of local inoculation was of relatively short duration and terminated with spontaneous resolution.

It was found that while a good growth could be obtained with material which had been subjected to repeated freezing, thawing, and grinding, the use of cell-free filtrates or desiccated tumor tissue gave negative results.

In discussing the experiments reported, it was pointed out that there were three groups of factors which had played important parts in determining the results of transplantation. These were the capacity for growth of the tumor cells, animal resistance, and the advantage given the tumor or the animal by the use of a particular method of inoculation. From an analysis of the results obtained with reference to the operation of these factors, the conclusion was reached that while the quality of malignancy displayed by the tumor was conditioned upon the capacity for growth of the tumor cells, the degree of malignancy exhibited in any given instance was a function of animal resistance which was not entirely specific but corresponded closely with the ability of the animal to meet excess demands arising from other causes and, to this extent, represented an expression of the functional capacity of a mechanism which is concerned primarily with the maintenance of the general animal economy.

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3. Rous, P., *J. Am. Med. Assn.*, 1911, lvi, 198. Rous, P., and Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 52.

EXPLANATION OF PLATES.

All the illustrations are reproductions of photographs which have not been retouched. Objects are represented at their natural size. Statements of time are estimated from the day of inoculation.

Plate 44.

Fig. 1. 9 days. Actively growing primary tumor in the subcutaneous tissues with marked enlargement of the inguinal lymph nodes.

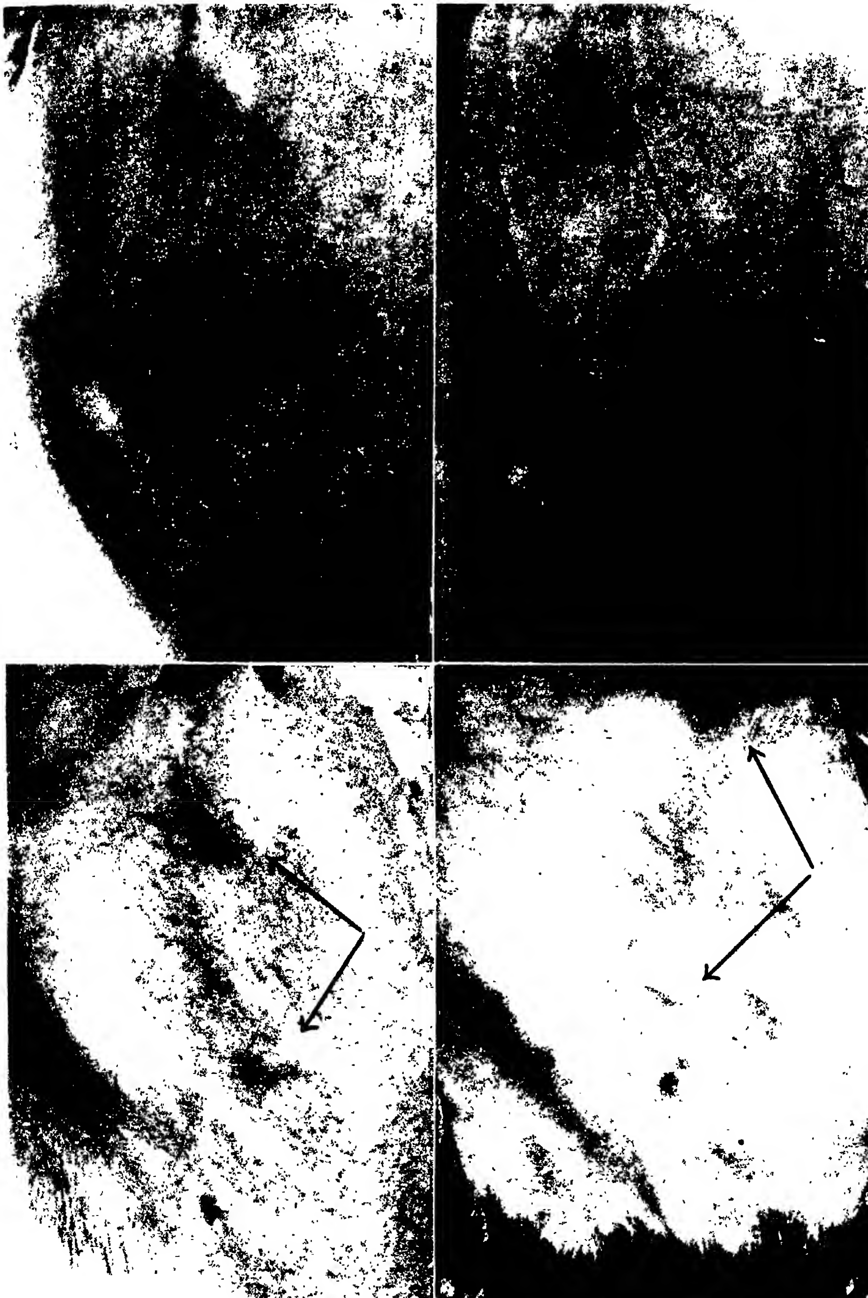
Fig. 2. 9 days. A flattened type of primary growth in the subcutaneous tissues, comparable in all other respects with that in Fig. 1.

Fig. 3. 9 days. A small nodular growth in the subcutaneous tissues of the upper mammary region with enlargement of the mammary and axillary lymph nodes.

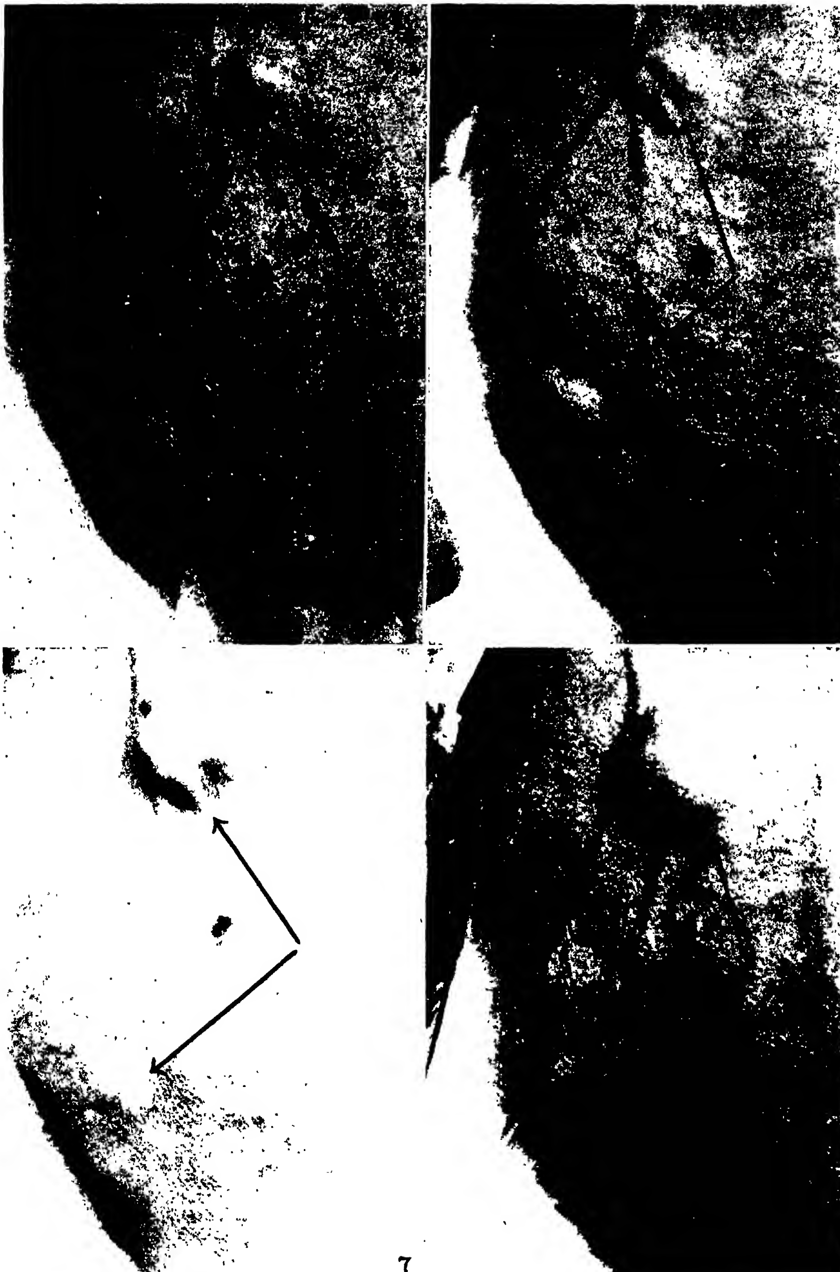
FIG. 4. 9 days. A negative result in an animal of the same series as that in Fig. 3. Note the small nodule of fibrous tissue at the site of inoculation (marked by an arrow) and the absence of any marked enlargement of the drainage lymph nodes.

PLATE 45.

FIGS. 5 to 8. 9, 14, 23, and 37 days respectively. Stages in the development of an unusually large and persistent tumor in the subcutaneous tissues. Note the relatively slow growth during the first 2 weeks as compared with the growth during the 3rd week and the reduction in the size of the inguinal lymph nodes between the 3rd and 5th weeks. The slow initial growth of the tumor may also be seen by comparing Figs. 1 and 5.



(Pearce and Brown: Malignant tumor of the rabbit. IV.)



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(Pearce and Brown: Malignant tumor of the rabbit. IV.)

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SEPARATION OF THE TOXINS OF BACILLUS DYSENTERIÆ SHIGA.

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In 1920, Olitsky and Kligler[1] separated the poison of *Bacillus dysenteriæ* Shiga into two physically and biologically distinct toxins. One, a true, soluble, exotoxin, an early product of the growth of this microorganism *in vitro*, is relatively heat-labile and yields an antiexotoxic immune serum. The other, an endotoxin, a product of the autolysis, or disintegration, of the bacillus with the resultant liberation of intracellular components, is heat-stable and is not neutralized by antiexotoxic serum. The exotoxin has a specific affinity for the central nervous system in the rabbit; the endotoxin, on the other hand, affects only the intestinal tract.

Since the exotoxin arises in the early period of growth, and since the endotoxin results from bacterial disintegration—a factor difficult to control in growing cultures—there is great technical difficulty in preparing pure endotoxin or exotoxin directly from the Shiga bacillus. Olitsky and Kligler have indicated methods for their separation. In this paper we shall describe an additional method which we believe to be more simple and effective. In this way the previous observation that the toxins of this microorganism are distinct has been confirmed.

It is relatively more simple to obtain pure neurotoxic exotoxin. Merely the filtrate of the early growths of Shiga bacilli, before bacterial disintegration occurs, suffices. Hence young, vigorously growing, well oxygenated cultures, incubated at 37°C. for from 1 to 3 or even to 5 or 7 days, depending on the rate of autolysis, or disintegration, when filtered through Berkefeld V or N candles, yield pure exotoxin in the bacteria-free filtrate.[1] This is not the case, however, with

[1] Olitsky, P. K., and Kligler, I. J., *J. Exp. Med.*, 1920, xxxi, 19.

endotoxin. The endotoxin which arises later as a result of bacterial dissolution is always admixed with the exotoxin which is first produced in the medium, and therefore the toxins require mechanical separation for purification.

Suppression of Exotoxin by Anaerobiosis.

If the exotoxic function of Shiga bacilli could be suppressed, the problem of producing pure endotoxin directly from disintegrated bacilli would be simplified. In seeking for a method for this suppression, use was made of the underlying principle of rapid and increased exotoxic production—oxygenation. Roux and Yersin,[2] by passing a stream of oxygen through the cultures or employing thin layers of culture fluid, determined the requirements for the prompt and effective production of diphtheritic soluble toxin—a method subsequently yielding successful results in the hands of Park and Williams[3] and accepted generally as a standard, not only for the diphtheritic exotoxin but also for that of other bacteria, including the Shiga bacillus.[1,4] If the converse held, that is, if Shiga bacilli were grown in an oxygen-free atmosphere and in a deep layered medium, the exotoxic function would be lowered or destroyed so that mechanical disintegration of the bacterial bodies in this medium might yield pure endotoxin.

In 1904, Rosenthal[4] found that anaerobic conditions diminish the yield of Shiga bacillus poison, although no mention was made of which type of the toxin is involved. Later, Doerr[5] affirmed that under similar conditions no specific dysenteric toxin is present in filtrates. No reference was made by these investigators, however, to any toxin and its type resident in the bacterial bodies comprising the residuum.

Experiments with Aerobic Cultures.

Our first attempts at experimentation related to the study of the toxicity of the particular strain of *Bacillus dysenteriæ* Shiga employed,

[2] Roux, E., and Yersin, A., *Ann. Inst. Pasteur*, 1888, ii, 629; 1889, iii, 273.

[3] Park, W. H., and Williams, A. W., Pathogenic microörganisms, Philadelphia and New York, 7th edition, 1920, 342.

[4] Rosenthal, L., *Deutsch. med. Woch.*, 1904, xxx, 235.

[5] Doerr, R., Das Dysenterietoxin, Jena, 1907, 30.

Strain 109.[1] This culture was maintained in artificial media for 5½ years, or 3½ years after having been tested by Olitsky and Kligler.[1]

Experiment 1.[6]—2 per cent peptone broth was placed in a Noguchi leptospira vaccine flask—a flask made of Pyrex glass measuring 10 cm. in height, with a diameter at the neck of 1.5 cm. and at the base of 7.5 cm. The object in using this sort of vessel was to allow thorough aeration, by providing a large surface area. Sufficient medium was employed to make a layer of broth 0.5 cm. deep. The broth was adjusted to pH 7.4. We desired to avoid an alkaline reaction since it encourages autolysis, or disintegration, of the bacilli, thus liberating the intracellular endotoxin. The medium was inoculated with a loopful of *B. dysenteriæ* Shiga, Strain 109, obtained from a plain agar slant growth of 18 hours duration. The flask was incubated for 3 days at 37°C. and frequently shaken to aerate the contents. The culture was then centrifuged and the supernatant fluid decanted and filtered through Berkefeld V candles. The bacteria-free filtrate was injected into the auricular vein of rabbits weighing about 1,800 gm.

The rabbits which received 1, 2, 5, and 10 cc. died, depending on the dosage, within 5 to 24 hours, with symptoms indicative of lesions in the medulla and upper cervical cord. These lesions were noted in stained sections and consisted of extensive hemorrhages, chiefly, along with edema, necrosis, and changes in neurons already described by Olitsky and Kligler.[1] A rabbit injected with 0.4 cc. showed no symptoms until the 4th day after injection, when complete paralysis occurred in the anterior extremities with paresis of the posterior limbs, and complete paralysis of all the extremities on the 5th day. The nervous symptoms endured for 4 days; thereafter the animal returned slowly to normal. A rabbit injected with 0.35 cc. of the filtrate exhibited slight weakness of the posterior extremities 4 days after inoculation, which lasted for 1 day only. In none of all these rabbits injected with the filtrate were any intestinal symptoms noted.

At the same time, the centrifuged, sedimented bacilli were washed in sterile distilled water, recentrifuged, and the resultant deposit suspended in 5 cc. of distilled water, to which 1 per cent of sodium carbonate had been added. The mixture was then heated for 30 minutes at 56°C. and kept for 24 hours at 37°C., so as to favor autolysis. Prior to injection, the autolysate was examined by means of Gram's stain and numerous shadow forms of the bacilli were seen. Cultivation tests demonstrated that the bacilli were dead.

A rabbit injected with 0.1 cc. of the autolysate of Shiga bacilli showed 4 days after inoculation a transitory mucous diarrhea of 2 days duration from which it promptly recovered. There were no signs of nervous involvement. A rabbit inoculated with 0.5 cc. of the autolysate presented paralysis of the anterior extremities after 24 hours and died after 48 hours. Autopsy revealed the typical nervous

[6] Only typical protocols are presented of a number of repeated experiments.

lesions, together with a glazed appearance of the intestines, which were edematous and congested, and the intestinal contents gave a positive benzidine test for blood. Histologically, the appearances corresponded to those already described by Flexner and Sweet,[7] Doerr,[5] Dopter,[8] and others,[1] as a result of the action of Shiga bacillus toxin.

This experiment was repeated, employing, instead of a 3 day growth of Shiga bacilli in Noguchi flasks, a 24 hour growth. The results were practically identical; the dosage of the bacteria-free filtrate required to induce nervous lesions without intestinal involvement and the dosage of the autolysate of the bacilli necessary to produce intestinal lesions combined with nervous lesions were the same as in the case of the 3 day growth.

From the above experiment it will be noted that Shiga bacilli, Strain 109, when grown in a well aerated medium, forms in 1 to 3 days a soluble toxin which is recoverable in bacteria-free filtrates. This exotoxin exerts a typical action on the central nervous organs of the rabbit, producing mainly hemorrhages and necroses in the medulla and upper cervical cord, but not affecting the intestinal tract. On the other hand, autolysates of the bacterial bodies induce not only similar nervous lesions but also intestinal involvement. Therefore exotoxin, or neurotoxin, a product of the growth of Shiga bacilli, can be obtained in a pure state by this method; but endotoxin, or enterotoxin, the result of bacterial disintegration, is always admixed with a certain amount of exotoxin.

Experiments with Anaerobic Cultures.

The next experiments concerned the complete suppression of the neurotoxin-producing activity of Shiga bacilli by employing anaerobic methods and a study of the disintegration products of the bacterial bodies in such cultures.

Experiment 2.—For producing anaerobic conditions either of two methods, both giving practically the same results, was employed. The first method consisted in the inoculation of a tall column (15 cm.) of 1 per cent dextrose broth, pH 7.4, in a Noguchi test-tube, with a loopful of Shiga bacilli, Strain 109, grown pre-

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[7] Flexner, S., and Sweet, J. E., *J. Exp. Med.*, 1906, viii, 514.

[8] Dopter, M. C., Les dysenteries, Paris, 1909, 75 ff.

viously for 18 hours in Brown's anaerobic jar.[9] The inoculated tubes were then placed in a Brown jar and incubated for 24 hours at 37°C. The growth of this tube was then subplanted into another and kept under similar anaerobic conditions. In this way the Shiga bacilli were subplanted to four consecutive anaerobic cultures, each incubating for 24 hours at 37°C. The fourth subplant was employed in the following experiment. The other method for obtaining anaerobic conditions consisted of layering over a similar column of the broth with petrolatum, then placing in an Arnold sterilizer and heating for ½ hour at 100°C. The medium was now anaerobic and was inoculated through the seal with 0.1 cc. of a Shiga bacillus broth culture grown previously for 18 hours in a Brown anaerobic jar. The inoculated, sealed tubes were kept for 24 hours at 37°C. With these exceptions, the method corresponded exactly with the one in which the Brown jar replaced the petrolatum seal for maintaining anaerobic conditions.

These anaerobic cultures were centrifuged at high speed until the supernatant fluid was clear. The latter was decanted, filtered through Berkefeld V or N candles, and the bacteria-free filtrate inoculated into the auricular vein of rabbits.

Rabbits which received 0.5, 1, 2, and 4 cc. of this anaerobic bacteria-free filtrate were unaffected and remained free from nervous or intestinal symptoms for a period of observation lasting 2 weeks or more.

The centrifuged, sedimented bacilli of these anaerobic cultures were, at the same time, washed in sterile distilled water, recentrifuged, and the clear supernatant fluid, or washings, decanted. The solid sediment of bacilli was suspended in 5 cc. of distilled water to which 1 per cent of sodium carbonate had been added. The mixture was heated for 30 minutes at 56°C. so as to kill the microorganisms, and then kept for 24 hours at 37°C. to favor autolysis. Prior to injection the autolysate was stained by Gram's method and cultured, and the mixture was found to consist of either completely autolyzed or dead bacilli. This material, in doses of 0.1, 0.2, 0.25, and 0.5 cc., was injected into the auricular vein of rabbits. The animals showed pronounced intestinal symptoms from 24 to 48 hours after inoculation, depending on the dosage. The first sign to be noted was straining and tenesmus, which was continual, accompanied by frequent evacuations of small, semisolid masses of feces. As the condition progressed, there was a mucous diarrhea streaked with either visible or microscopic blood, determined by the benzidine test. This condition persisted for 2 to 5 days, the animals returning, thereafter, to normal. Indeed, none of this series died. Some of the animals were killed at the height of the reaction for macroscopic and histopathological examination. The intestines then revealed grossly the typical glazing of the peritoneal coat, the edema, the injection of blood vessels, and hemorrhages, and showed microscopically the edema, hemorrhages, necroses, ulceration, and fibrinous (diphtheritic) covering of the epithelial coat, corresponding to the description given by Flexner and Sweet,[7] Doerr,[5]

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[9] Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677; 1922, xxxv, 467.

and Dopter[8] of the action of the Shiga poison, and later by Olitsky and Kligler[1] of the effects of Shiga endotoxin. In none of these animals were noted any signs of nervous involvement, either clinically or in section, even when ten times (1 cc.) of the minimal enterotoxic dose was used.

This experiment, typical of four similar ones, demonstrates that the exotoxic function of Shiga bacilli can be suppressed by growing the microorganisms under anaerobic conditions, either in fluid medium under a petrolatum seal or in this medium placed in an anaerobic jar. The bacteria, now in an exotoxin-free environment, can be autolyzed, or dissolved, with the resultant liberation of intracellular components, or endotoxin. The endotoxin, the preparation of which does not differ essentially from that of endotoxins from other bacteria, has a specific affinity for the intestinal tract, producing edema, hemorrhages, necroses, and ulcerations. The central nervous organs are not affected; hence the endotoxin can be properly designated as an enterotoxin.

We have thus separated the two toxins of the strain of *Bacillus dysenteriæ* Shiga which has been employed and have shown their biological distinctions. We now proposed to extend the observations of Olitsky and Kligler[1] by studying additional physical differencse.

Diffusion of Toxins through Permeable Membranes.

The diffusion of the toxins through the walls of collodion sacs was investigated.

Methods.—Permeable collodion sacs for intraabdominal implantation and those for bacterial cultivation *in vitro* were prepared, following closely Gates' method.[10,11] For intraperitoneal implantation, the sacs were made on No. 12 size veterinary capsules;[10] for cultivation *in vitro*. the sacs were prepared in flasks.[12]

Preliminary experiments resulted in the choice of 2 per cent peptone broth (pH 7.4, for the reasons stated above) instead of the egg albumin broth which was suggested by Olitsky and Kligler[1] as the most effective medium. While the contents of the sac containing cultures of Shiga bacilli in the albumin broth were highly

[10] Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 25.
[11] Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 635.
[12] Gates,[11] p. 640.

toxic for rabbits, dialysates contained less toxin than that obtained by the use of a clear simple medium such as the peptone broth. The dense cultures of the bacilli in albumin broth coat the inner surface of the sac, thus preventing active dialysis.

Experiments with Sacs in Vivo.—Five rabbits were employed and into the abdominal cavity of each were placed two sealed sacs containing the broth inoculated with two loopfuls of agar slant cultures of Shiga bacilli. Four of the rabbits were unaffected for a period of 1 month. Then the sacs of two of the animals were removed and the contents were examined. A growth of Shiga bacilli was obtained on transplanting to agar, although a number were found autolyzed in stained preparations of the material. The contents of the sacs from one rabbit were reinjected into the auricular vein of two normal animals in doses of 0.1 and 0.05 cc. These latter died in 48 hours with typical nervous and intestinal symptoms. One of the rabbits containing the sacs was also injected at the same time with 0.1 cc. of the sac contents. This animal was unaffected. The experiment was repeated with similar results.

One of the four rabbits unaffected by harboring the sac cultures showed, 1 month after implantation, an agglutination serum titer of 1:1,280 against Shiga bacilli. The remaining three were negative.

The fifth rabbit exhibited, 5 days after implantation of the sacs, paralysis of both anterior extremities, paresis of the posterior extremities, and hyperesthesia. There were no intestinal symptoms. The animal died the next day, showing complete paralysis but no intestinal symptoms. The histopathology revealed the typical nervous lesions but the intestinal tract was normal. That these effects were not due to breakage of the sac and liberation of the bacteria was proved by culturing the sealed sacs after removal from the body for 48 hours in dextrose broth. No growth of Shiga bacilli occurred in the surrounding medium.

From these experiments it may be concluded that the Shiga bacilli, when grown in fluid medium contained in a sac which is implanted intraabdominally, survive and multiply therein for at least 1 month. During this period different effects are produced, depending, we believe, on the state of permeability of the sac. Rabbits harboring highly permeable sacs show the results of dialysis of pure exotoxin within 5 days after implantation; that is, the animal succumbs to lesions produced in the medulla and upper cervical cord. One may assume that if the exotoxin would dialyze out of the sac in nonlethal amounts the rabbit might exhibit at a later time intestinal symptoms due to subsequent disintegration of the bacilli within the sac with formation of endotoxin. Rabbits containing sacs of a lesser degree of permeability, although sufficient to allow an active diffusion

of the body fluids through their walls, produce a high titer of agglutinins in their blood sera. In this respect we have been able to confirm a previous observation of Gates,[13] who found that typhoid bacilli survive and multiply in sacs *in abdomine* for 1½ years; during this time agglutinins for these bacilli are produced. Even when agglutinins are not detectable in the serum of these animals, they remain immune to a later injection of either exotoxin or endotoxin.

The difficulty of determining the fine degrees of permeability of the sacs necessary to induce these effects renders this method unsatisfactory for our purposes of separating the two dysenteric toxins. However, certain conclusions may be drawn from these experiments: The contents of the sacs after 1 month *in abdomine* are highly toxic for normal rabbits, which show the effects of both exotoxin and endotoxin, corresponding to the multiplication and autolysis and dissolution proceeding in the sacs. Exotoxin diffuses through, primarily causing the death of the animal by the typical nervous lesions. Agglutinins are produced in surviving animals, in one instance in a titer of 1:1,280. Finally, immunity is conferred in the latter to subsequent injections of exotoxin and endotoxin.

Experiment with Sacs in Vitro.—For the purposes of these experiments, Gates' method of preparing sacs in flasks[13] was employed. 30 cc. of the same medium employed in the intraabdominal sacs were placed within the sac and 30 cc. of distilled water without. The medium within the collodion membrane was inoculated with two loopfuls of Shiga bacilli, Strain 109, grown for 18 to 24 hours on agar slants. The cultures were then kept for varying periods of time (from 3 to 8 days) at 37°C.

The cultures which were incubated 3, 4, and 5 days produced in the dialysate the typical exotoxin which induced in normal rabbits neurotoxic symptoms but no intestinal involvement. A single protocol is presented to show the manner in which an experiment was conducted and the results obtained. The protocol relates to one of a series of experiments made with a 5 day culture. The cultures kept at 37°C. for 3 and 4 days gave practically the same results, except that the nervous symptoms were of a milder degree and more transitory, enduring for 2 to 3 days, the animal returning thereafter to normal.

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[13] Gates, F. L., personal communication.

Experiment 3. 5 Day Culture.—

Rabbit.	Material injected intravenously.	Dose.	Symptoms and course following injection.
		cc.	
A	Dialysate.	10	48 hrs., paresis of right anterior extremity. 72 hrs., paralysis of both anterior extremities, hyperesthesia; paresis of posterior extremities. 4 days, condition unchanged. 5 days, condition improved; partial paralysis of anterior extremities; paresis of posterior extremities. 10 days, practically recovered. No intestinal symptoms during the 10 days.
B	"	10	48 hrs., hyperesthesia, paresis of posterior extremities. 4 days, paresis of anterior and posterior extremities. 7 days, partial paralysis of right anterior extremity; other limbs recovered. 11 days, practically recovered. No intestinal symptoms during the 11 days.
C	" + polyvalent antidysenteric serum* (injected simultaneously).	10 5	No effect.
D	Dialysate + polyvalent antidysenteric serum* (injected simultaneously).	10 5	" "
E	Dialysate heated at 75°C. for 1 hr.	10	" "
F† (immune)	Dialysate injected into rabbit which recovered from exotoxic paralysis 3 wks. previously.	10	" "

Rabbit.	Material injected intravenously.	Dose.	Symptoms and course following injection.
		cc.	
G	Contents of sac.‡	2	24 hrs., paresis of right anterior extremity; tenesmus. 48 hrs., paralysis of anterior extremities; paresis of posterior extremities; mucous diarrhea. 72 hrs., complete paralysis; diarrhea. Moribund; chloroformed. Nervous and intestinal lesions typical.

* This serum was the only therapeutic serum available. It was the Rockefeller Institute serum and contained exotoxic, endotoxic, and bacterial antibodies.[1]

† In another series of similar experiments with 5 day cultures, four animals which had been injected 2 to 3 weeks previously with exotoxin and had recovered from its action were reinjected with dialysate. They were all unaffected.

‡ On microscopic examination, the sac contents showed evidence of bacillary disintegration.

These experiments, which were repeated with similar results, demonstrate that the exotoxin is the first to appear in the bacteria-free dialysate. The exotoxin was identified by its power to induce nervous lesions, by its relative thermolability,[1] and by its failure to act in animals previously recovered from exotoxic effects (immunity tests), or when the toxin was injected simultaneously with immune serum. In the animals injected with the dialysates, no intestinal signs were observed, although on the 5th day the contents of the sac showed beginning autolysis, or disintegration, of the Shiga bacilli, and, consequently, caused both nervous and intestinal symptoms when injected into rabbits. However, after this period, and corresponding with an increasing degree of dissolution of the bacilli within the collodion membrane, both exotoxin and endotoxin appeared in the dialysates, as the following protocol shows.

Experiment 4. 6 to 8 Day Cultures.—Cultures such as were employed in Experiment 3 were allowed to incubate for 6, 7, and 8 days at 37°C. Normal rabbits were then injected with the different components of the cultures, as represented in the following protocol illustrating an experiment with a 6 day culture.

Rabbit.	Material injected intravenously.	Dose.	Symptoms and course following injection.
		cc.	
A	Dialysate.	10	24 hrs., paresis; mucous diarrhea. 48 hrs., paralysis and diarrhea. 72 hrs., completely paralyzed. Killed. Lesions in nervous and intestinal organs typical.
B	"	5	24 hrs., paresis of left anterior extremity and posterior extremity. 48 hrs., condition unchanged for 7 days thereafter. During the 9 days, no intestinal symptoms observed.
C	" heated for 1 hr. at 75°C.	10	Showed no nervous symptoms for 9 days; mucous diarrhea 3 days after injection which lasted 3 days; animal recovered thereafter.
D	Sac contents (containing numerous shadow forms along with viable Shiga bacilli).	1	24 hrs., paresis; mucous diarrhea. 48 hrs., paralysis of anterior extremities; paresis of posterior extremities; tenesmus; mucous diarrhea. Moribund; killed. Lesions in medulla, upper cervical cord, and intestines typical.

Experiments with 7 and 8 day cultures gave similar results, except that it required only 5 cc. of the dialysate to induce intestinal symptoms.

It appears from Experiment 4, therefore, that *in vitro* cultures of Shiga bacilli in collodion sacs produce mixtures of exotoxin and endotoxin in the bacteria-free dialysates. Hence, the early growths, that is up to 5 days incubation, yield pure exotoxin (Experiment 3) which diffuses readily through the collodion membrane and is detectable in the dialysate. After this period not only exotoxin but endotoxin diffuses through and the rate of the latter is proportional to the amount of dissolution, or disintegration, of the Shiga bacilli within the membrane. From the foregoing experiment it will be noted, as well, that the thermostable endotoxin in the dialysate can be separated from the relatively thermolabile exotoxin by heat.[1]

DISCUSSION.

Additional methods are presented to support the observations of Olitsky and Kligler[1] concerning the dual nature of the poison of Shiga bacilli. The first method consists of the suppression of the exotoxic, or neurotoxic, function of the microorganisms by anaerobiosis so that the disintegration of the bacteria, with consequent liberation of intracellular components, yields pure endotoxin, or enterotoxin. Thus the difficulty of securing pure endotoxin directly from the culture medium is overcome, for hitherto this toxin was always found admixed in the medium with exotoxin, which is an early product of the growth of Shiga bacilli.

The second method relates to the diffusion of these toxins through the walls of collodion sacs implanted intraabdominally or placed in flasks. Cultures in sacs implanted intraabdominally show different effects, depending on the permeability of the collodion membrane; highly permeable sacs allow the early passage of exotoxin, somewhat denser membranes permit only the diffusion of substances inducing agglutinins or immunity. Cultures in sacs placed in flasks yield in the fluid surrounding the membrane first exotoxin and later endotoxin. The passage through the membrane of endotoxin depends on the rate of bacterial disintegration within the sac.

Furthermore, the biological and physical differences of the two toxins which have already been demonstrated[1] are confirmed.

A practical bearing of this work concerns the preparation of an effective Shiga antidysenteric serum. The experimental production of antiexotoxin has already been described.[1] A potent serum for general use, however, should be anti-infectious and should contain endotoxic and exotoxic antibodies. The injection intravenously in horses of live Shiga bacilli, after the manner described by Flexner and Amoss,[14] has yielded a serum which contains 2,000 antiexotoxic units per cc., as well as endotoxic and bacterial antibodies (0.01 cc. of the serum neutralizes four minimal endotoxic doses and shows agglutinins ranging from 1:2,000 to 1:20,000 against Shiga bacilli[1]). Its anti-infectious potency as measured by Flexner and Amoss is such that 0.008 cc. protects rabbits against two lethal doses of Shiga bacilli.

[14] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1915, xxi, 515.

CONCLUSIONS.

By the suppression, through anaerobiosis, of the exotoxin-producing activity of *Bacillus dysenteriæ* Shiga a pure endotoxin is produced directly from the culture. The duality of the poison of Shiga bacillus is further substantiated by studies on the diffusion of exotoxin, or neurotoxin, and endotoxin, or enterotoxin, by means of collodion sacs, implanted intraabdominally in rabbits or placed *in vitro*.

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THE VIRULENCE OF AN EPIDEMIC STRAIN OF BACILLUS PESTIS CAVIÆ.

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Certain experiments described in an earlier paper[1] indicated that the virulence of a given strain of mouse typhoid bacilli[2] for mice bred at The Rockefeller Institute is relatively constant. But although this work was done according to the usual bacteriological technique, it was felt at the time that any cultivationon artificial media between animal passages introduced a factor which might affect the infecting power of the various cultures. Consequently, the following experiments were devised to eliminate cultivation on artificial media and to determine the effect of animal passage on the virulence of a native pathogenic strain.

Experiment 1.—This experiment was planned to determine the effect of direct intraperitoneal mouse passage, without intermediate cultivation in artificial media, on the virulence of this strain of mouse typhoid bacilli.

Technique.—An 18 hour broth culture from the current stock strain was diluted 1:1,000 and injected intraperitoneally into ten mice weighing 16 to 18 gm. each. 24 to 48 hours later, when the animals began to show morbid symptoms, blood cultures were taken from the tail vessels. On the following day, the mice with positive blood cultures were anesthetized and bled from the heart. About 0.5 cc. of blood per mouse thus obtained was collected in salt solution containing 2 per cent sodium citrate. This suspension was immediately injected intraperitoneally into another series of ten mice. Coincident with the seventh direct passage in this manner, 1 cc. of the suspension was diluted and plated in the usual way to estimate the number of bacteria per cubic centimeter. Also control intraperitoneal titrations were run with the current stock strain used in the first passage series and with the original stock strain which had had no animal passage for over 3 years.

[1] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 231.

[2] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.

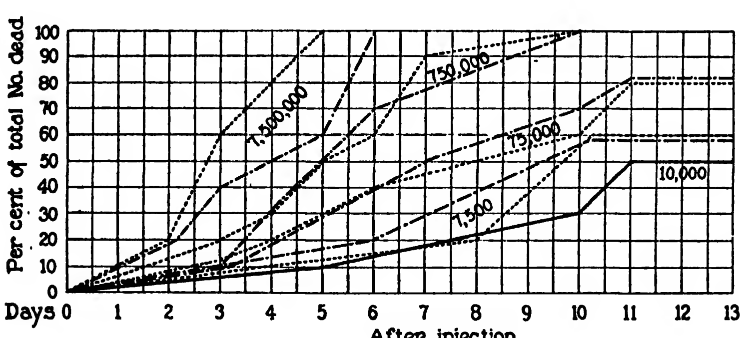
Table I and Text-fig. 1 compare the virulence of the three cultures. The mortality curves of the original culture and the current culture are quite similar for each dilution; the mortality curve of the "seven passage" mice, each of which received about 10,000 bacteria, compares with the curves of the control mice which received 10,000 and

TABLE I.

Duration of Life of Experimental and Control Mice Used in the Intraperitoneal Seventh Passage Test.

No. of mice.	Culture.	No. of bacteria injected.	Duration of life after injection.
			days
5	Original.	5,000,000	2, 3, 3, 4, 5
10	"	500,000	3, 3, 4, 5, 5, 6, 7, 7, 7, 10
10	"	50,000	3, 4, 5, 6, 8, 10, 11, 11*
5	"	5,000	8, 10, 10*
5	Current.	10,000,000	2, 3, 5, 6, 6
10	"	1,000,000	3, 4, 4, 5, 5, 6, 6, 7, 10, 10
10	"	100,000	4, 5, 7, 7, 7, 10, 10, 11*
5	"	10,000	6, 8, 10*
10	"Seven passage."	10,000	5, 10, 10, 11, 11*

* This test was terminated on the 13th day.



...... Original stock culture.
—· —· Current stock culture.
——— Current stock culture plus seven direct mouse passages.

TEXT-FIG. 1. The virulence of a strain of mouse typhoid bacilli after seven direct intraperitoneal mouse passages. To simplify the graph, the average number of bacteria per mouse in the two control series is given.

5,000 bacteria each, respectively. The test shows, then, that the virulence of this "seven passage" culture remains the same as that of the current stock culture and the original culture.

In order to continue the passage, this test was terminated on the 13th day. With the ninth direct passage, however, the series was allowed to run for 30 days, and a parallel control titration was employed again, using the current stock culture.

Table II and Text-fig. 2 show that the mortality rate of the "nine passage" mice, each of which received 1,000,000 bacteria, is about the same as the mortality rate of the control mice which received a similar number of bacteria of the current culture. It may be concluded,

TABLE II.

Duration of Life of Experimental and Control Mice Used in the Ninth Passage Test.

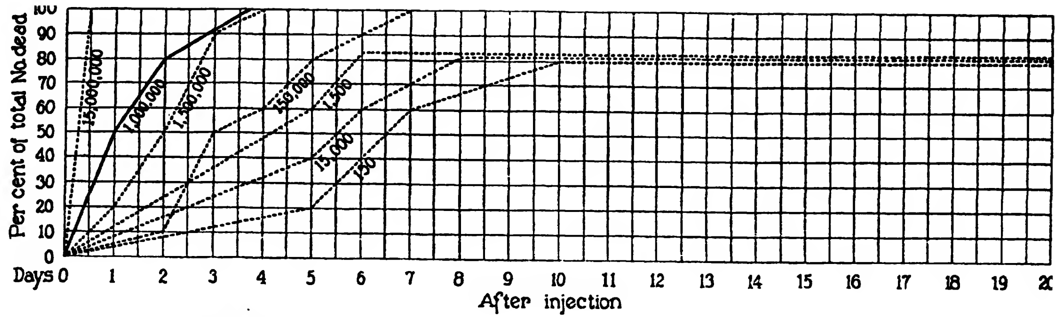
No. of mice.	Culture.	No. of bacteria injected.	Duration of life after injection.
			days
5	Current.	15,000,000	1, 1, 1, 1, 1
10	"	1,500,000	1, 1, 2, 2, 2, 3, 3, 3, 3, 4
10	"	150,000	2, 3, 3, 3, 3, 4, 5, 5, 6, 7
5	"	15,000	5, 5, 6, 8, S.*
5	"	1,500	5, 5, 5, 6, S.*
5	"	150	5, 6, 7, 10, S.*
10	"Nine passage."	1,000,000	1, 1, 1, 1, 1, 2, 2, 2, 3, 4

* Survived 30 days.

therefore, that nine direct intraperitoneal mouse passages with no intermediate cultivation on artificial media do not affect the virulence of this strain of mouse typhoid bacilli.

Experiments 2 and 3 were planned to determine the effect of direct *per os* mouse passages, without intermediate cultivation in artificial media, on the virulence of this strain of mouse typhoid bacilli. In Experiment 2, the bacterial suspensions were obtained from the heart's blood and spleen of animals in the acute stages of infection; in Experiment 3, the material was obtained from the intestinal contents of similar animals.

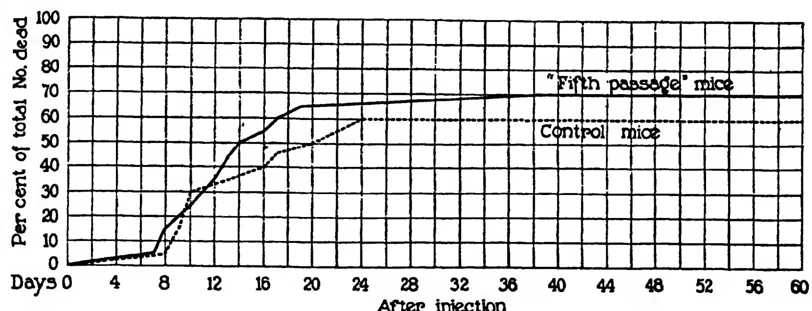
Experiment 2.—100 mice from the breeding room were given *per os* by stomach tube an 18 hour broth culture from the current stock strain, diluted 1:200. 9



........Current stock culture.

————Current stock culture plus nine direct mouse passages.

TEXT-FIG. 2. The virulence of a strain of mouse typhoid bacilli after nine direct intraperitoneal mouse passages.



TEXT-FIG. 3. The virulence of a strain of mouse typhoid bacilli after five direct *per os* mouse passages (Experiment 2).

days later, 5 mice had died and 30 of the survivors showed positive blood cultures. These 30 mice were anesthetized, and for Experiment 2, 0.5 cc. of heart's blood from each mouse was put into salt solution containing 2 per cent citrate, and the spleen and heart were emulsified in 0.8 per cent salt solution in a sterile mortar. The two emulsions were combined. After slow centrifugation, the supernatant fluid was injected *per os* into the second series of 30 mice, each animal receiving 0.5 cc. of the suspension containing approximately 50,000,000 bacilli. This and all subsequent series were kept in 8 by 10 inch battery jars, 2 mice per jar. 11 days after the second series of 30 mice was injected *per os* with the blood and spleen suspension, 6 mice had died and 15 of the survivors showed positive blood cultures. From these 15 mice, heart's blood, heart, and spleen were taken, treated, and injected *per os*, as described above, into the third series of 30 mice. The number of bacilli given was this time about 5,000,000. 9 days later 6 mice had died. In the same manner, a blood and spleen emulsion was prepared from the survivors show-

TABLE III.

Duration of Life of Experimental and Control Mice Used in the per Os Blood and Spleen Experiment (Experiment 2).

Culture.	No. of bacteria injected.	No. of mice.	Duration of life after injection.
			days
"Fifth passage."	6,500,000	20	7, 8, 8, 9, 10, 12, 12, 13, 13, 14, 16, 17, 19
Control.	2,000,000	19	8, 9, 9, 10, 10, 10, 13, 16, 17, 20, 22, 24

ing positive blood cultures and given *per os* to the fourth series of 30 mice of which each received about 25,000,000 bacilli. 14 days later, 7 of these mice were dead and 12 showed positive blood cultures. A blood and spleen emulsion was prepared as before from the 12 mice and injected *per os* into the fifth series of 20 mice, each receiving about 6,500,000 bacilli. As controls, 19 mice were given a 24 hour broth culture from the stock agar tube diluted so that each mouse received approximately 2,000,000 bacilli.

Table III and Text-fig. 3 compare the duration of life of the series receiving the mouse passage culture with that of the series receiving the unpassed stock culture. The mortality curves are so nearly identical as to indicate that the "fifth passage" culture possesses about the same degree of virulence as does the control unpassed culture.

Experiment 3.—Of the first 100 mice used in Experiment 2, 30 showed positive blood cultures on the 9th day. The small and large intestines of these mice were emulsified in salt solution, centrifuged, and injected *per os* into a series of 30 mice, each animal receiving 0.5 cc. of the suspension. 11 days after this second series of 30 mice was injected, 6 were dead and 15 of the survivors showed positive blood cultures. From these 15 mice, intestine and stool suspensions were made as described above, centrifuged, and injected *per os*, 0.5 cc. per mouse, into the third series of 30 mice. 9 days later, 7 mice had died and 7 showed positive blood cultures. The above technique was repeated and the resulting stool suspension injected *per os* into the fourth series of 30 mice. 13 days later, 5 of these mice had died and 10 showed positive blood cultures. The intestines from these 10 survivors were ground with sand, emulsified, and injected in 0.5 cc. quantities into the fifth series of 20 mice. The controls employed in Experiment 2 were used for this series.

TABLE IV.

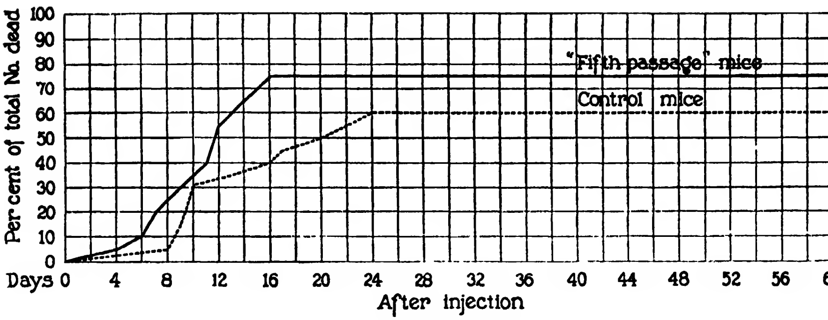
Duration of Life of Experimental and Control Mice Used in the per Os Stool Emulsion Experiment (Experiment 3).

Culture.	No. of bacteria injected.	No. of mice.	Duration of life after injection.
			days
"Fifth passage."	50,000,000*	20	4, 6, 7, 7, 8, 9, 11, 11, 12, 12, 12, 13, 14, 16, 16
Control.	2,000,000	19	8, 9, 9, 10, 10, 10, 13, 16, 17, 20, 22, 24

* This is merely a rough estimate based on spreading a measured amount of diluted stool suspension over green dye plates and counting the suspicious colonies after 24 hours incubation.

Table IV and Text-fig. 4 compare the duration of life of the series receiving the mouse passage culture with that of the series receiving the unpassed stock culture. The mortality curve of the mice receiving the stool suspension rises abruptly, in a manner similar to that observed when mice were sensitized with ox bile,[3] but the total mortality is not materially different from that of control mice. A rough estimation of the number of colonies per cubic centimeter in the stool suspension, determined by means of green dye plates, gave 50,000,000 per mouse in contrast to 2,000,000 per mouse in the control.

[3] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 33.



TEXT-FIG. 4. The virulence of a strain of mouse typhoid bacilli after five direct *per os* mouse passages (Experiment 3).

DISCUSSION AND SUMMARY.

Ever since Pasteur's demonstration of the modifying effect of animal passage on the virulence of bacteria, this device has been regarded as of great importance in intensifying infective capacity. It is not often, however, that extensive parallel tests have been made of the power of a microorganism to produce infection in a species of animal which is its natural habitat and under conditions in which the normal as well as artificial portal of entry is employed, and the potency of the microorganism passed directly from host to host is closely contrasted with that of one merely artificially cultivated outside the body.

The experiments described in this paper were arranged to fulfill these conditions. The results show that with the particular strain of *Bacillus pestis caviæ* used, successive animal passages do not modify infective capacity, or virulence. This finding is in harmony with the observations previously made from which it was concluded that the epidemic curve of mouse typhoid infection is explicable solely on the basis of bacillary distribution and host susceptibility.[4]

[4] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 269.

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MICROBIC VIRULENCE AND HOST SUSCEPTIBILITY IN PARATYPHOID-ENTERITIDIS INFECTION OF WHITE MICE. I.

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Earlier reports [1–3] on the study of mouse typhoid infection have been concerned with a single strain of bacilli. We wish now to describe a series of experiments on mice with six related strains of paratyphoid-enteritidis bacilli isolated from man and animals. In a first communication we propose to consider the effect of repeated intraperitoneal passage on the virulence of the several strains and in a second[4] to analyze the equilibrium between the virulence of each strain and the individual and group resistances of the various mice employed.

EXPERIMENTAL.

The mice were raised at The Rockefeller Institute in the breeding station already described.[2] Animals of one age and nearly uniform weight were chosen and fed on a diet of bread and milk. For reasons of economy of space and care two mice were placed in each 8 by 10 inch battery jar. The strains of paratyphoid-enteritidis bacilli used for inoculation have been described in earlier papers[2,3] and can be identified from their designations which follow: Mouse Typhoid I (M. T. I), *Bacillus enteritidis* (Gaertner), Mouse Typhoid II (M. T.

[1] Flexner, S., *J. Exp. Med.*, 1922, xxxvi, 9.

[2] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25, 45. Amoss, H. L., and Haselbauer, P. P., *J. Exp. Med.*, 1922, xxxvi, 107.

[3] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 71, 97; 1923, xxxvii, 21, 33, 231, 269.

[4] Webster, L. T., *J. Exp. Med.*, 1923, xxxviii, 45.

II), *Bacillus pestis caviæ*, *Bacillus aertrycke* (mutton), and *Bacillus paratyphosus* B.[5]

In dealing with these several strains in which the cultural and serological reactions are so nearly alike, particular care is required in order to avoid error. To emphasize this point, we have interpolated a table which summarizes the fermentation and agglutination reactions of the several strains and indicates their relative pathogenicity for mice (Table I).

Experiment 1.—The six strains grown on agar slants were transferred to broth, incubated for 18 hours, and then diluted as follows: 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000. Two mice were given intraperitoneally 1 cc. of each dilution. By the usual plating method, the number of bacteria per cubic centimeter in each dilution was estimated. All the mice succumbing were submitted to autopsy and cultures made from the heart's blood and spleen. The growths obtained were checked by tests outlined in Table I.

The identified broth culture from the heart's blood of the first mouse to die of each series after suitable dilutions was used to inject the next series of mice. Each of the six strains underwent from four to eight passages in this manner; control titrations were then made with the original unpassed strains taken directly from the stock agar tube.

Tables II and III illustrate the manner of recording duration of life of the mice used in each passage of every strain. Text-figs. 1 to 6 plot the results of the entire experiment in a convenient manner by showing the virulence of the given strain in five different dilutions for each consecutive passage. No progressive increase in virulence for each passage in any dilution of any strain or appreciable difference between the virulence of the passed culture and the control unpassed culture was observed. The occasional discrepancy in the otherwise uniform results will be accounted for later on the basis of differences in individual host susceptibility.

Text-fig. 7 compares the intraperitoneal virulences of the six strains in four different dilutions. It may be seen that in all dilutions,

[5] M. T. I is a mouse typhoid strain isolated from a spontaneous case and closely related culturally and serologically to *B. enteritidis* (Gaertner). M. T. II is the mouse typhoid strain employed throughout our series of studies. It has been identified with *B. pestis caviæ* and related very closely to *B. aertrycke* (mutton).

B. pestis caviæ, *B. enteritidis* (Gaertner), *B. aertrycke* (mutton), and *B. paratyphosus* B are stock strains obtained from Dr. Charles Krumwiede.

TABLE I.

Differentiation of Six Paratyphoid-Enteritidis Strains.

Strain.	Cultural reactions.								Agglutination titer in immune sera.					Pathogenicity for mice.
	Glucose.	Maltose.	Xylose.	Arabinose.	Lactose.	Saccharose.	Lead acetate reduction.	Inosite.	M. T. I.	M. T. II.	*B. pestis caviæ.*	*B. aertrycke* (mutton).	*B. paratyphosus* B.	
M. T. I.	+	+	+	+	−	−	+	−	1:10,000 ++	−	−	−	−	High.
B. enteritidis	+	+	+	+	−	−	+	−	+	−	−	−	−	"
M. T. II.	+	+	+	+	−	−	+	+	−	1:10,000 ++	+1	+1	+	Moderate.
B. pestis caviæ	+	+	+	+	−	−	+	−	−	+	1:3,000 ++	+	1 or −	Low.
" *aertrycke* (mutton)	+	+	+	+	−	−	+	−	−	+	+1	1:4,000 ++	+	Moderate.
" *paratyphosus* B	+	+	+	+	−	−	+	+	−	− or 1	−	−	1:3,000 ++	Low.

Agglutination test symbols: ++, complete agglutination to titer; +1, agglutination, cloudy supernatant fluid, to titer; +, fair agglutination to titer; 1, trace of agglutination to titer; −, no agglutination at titer.

TABLE II.

Intraperitoneal Passage of Strain M. T. I.

Passage No.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.
			days			*days*			*days*			*days*			*days*			*days*
2	19,000,000,000	2	1, 1	1,900,000,000	2	1, 1	190,000,000	2	1, 1	19,000,000	2	1, 20*	1,900,000	2	1, 3	190,000	2	1, 1
3	1,500,000,000	2	1, 1	150,000,000	2	1, 2	15,000,000	2	1, 2	1,500,000	2	1, 1	150,000	2	1, 2	15,000	2	4, 6
4	500,000,000	2	1, 1	50,000,000	2	1, 1	5,000,000	2	1, 1	500,000	2	1, 1	50,000	2	2, 3	5,000	2	2, 6
5	5,000,000	2	1, 1	500,000	2	1, 2	50,000	2	4, 4	5,000	2	2, 4	500	2	S., S.†			
6	4,000,000	2	1, 1	400,000	2	1, 2	40,000	2	3, S.	4,000	2	5, 6	400	2	11, 11			
7	4,000,000	2	1, 2	400,000	2	2, 2	40,000	2	2, 4	4,000	2	4, 4	400	2	4, 5			
8	3,000,000	2	1, 1	300,000	2	2, 3	30,000	2	2, 2	3,000	2	3, 3	300	2	5, 11			
C.	2,500,000	2	1, 2	250,000	2	2, 2	25,000	2	3, 6	2,500	2	2, 12	250	2	2, 8			

* Autopsy cultures negative.

† S. indicates that the animal survived for 30 days; C. indicates control.

TABLE III.

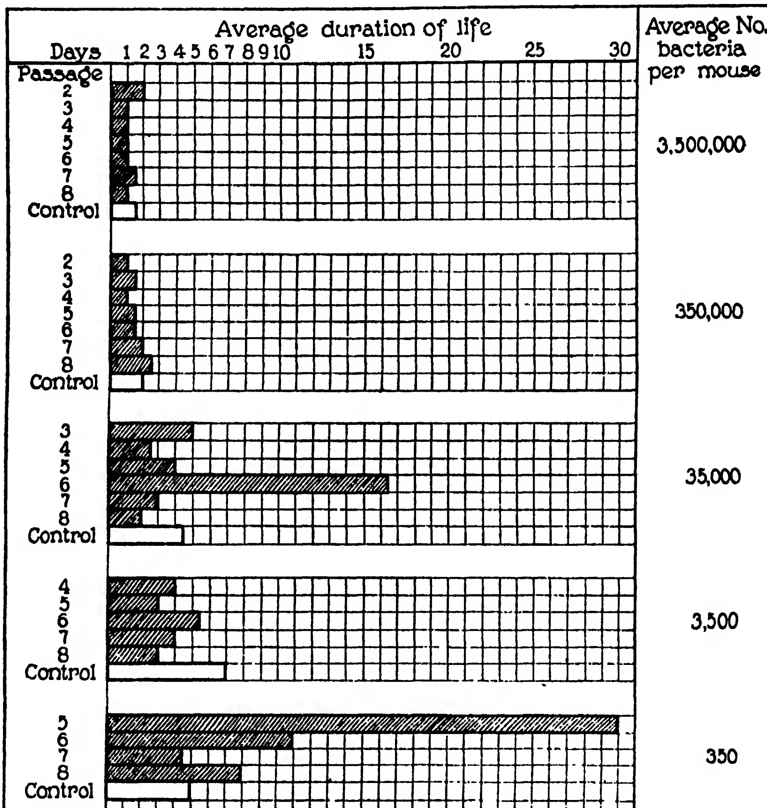
Intraperitoneal Passage of Strain B. pestis caviæ.

Passage No.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.	No. of bacteria per mouse.	No. of mice.	Duration of life.
			days			*days*			*days*			*days*			*days*			*days*
2	19,000,000,000	2	1, 1	1,900,000,000	2	1, 1	190,000,000	2	1, 1	19,000,000	2	1, 4	1,900,000	2	4, 7	190,000	2	6, 9
3	250,000,000	2	*1, 1	25,000,000	2	1, 1	2,500,000	2	1, 1	250,000	2	4, 6	25,000	2	6, S.	2,500	2	8, 27*
4	200,000,000	2	1, 1	20,000,000	2	1, 1	2,000,000	2	1, 2	200,000	2	3, 4	20,000	2	5, 6	2,000	2	6, 10
5	3,500,000	2	1, 2	350,000	2	3, 4	35,000	2	5, 6	3,500	2	S., S.†	350	2	S., S.			
6	2,500,000	2	2, 5	250,000	2	3, 5	25,000	2	2, 3	2,500	2	8, 12	250	2	" "			
7	3,000,000	2	2, 5	300,000	2	4, 4	30,000	2	4, 4	3,000	2	10, 11	300	2	" "			
8	2,000,000	2	3, 5	200,000	2	5, 13	20,000	2	5, 8	2,000	2	S., S.	200	2	" "			
C.	2,000,000	2	3, 3	200,000	2	1, 3	20,000	2	5, 6	2,000	2	8, "	200	2	25, "			

* Autopsy cultures negative.

† S. indicates that the animal survived for 30 days; C. indicates control.

Strains M. T. I and *Bacillus enteritidis* are most virulent and are quite similar; that Strains M. T. II and *Bacillus aertrycke* (mutton) have the same degree of virulence, which is less than the enteritidis

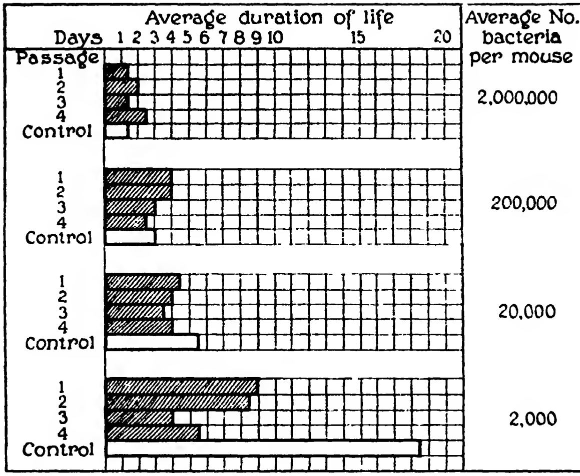


TEXT-FIG. 1. Intraperitoneal virulence of Strain M. T. I.

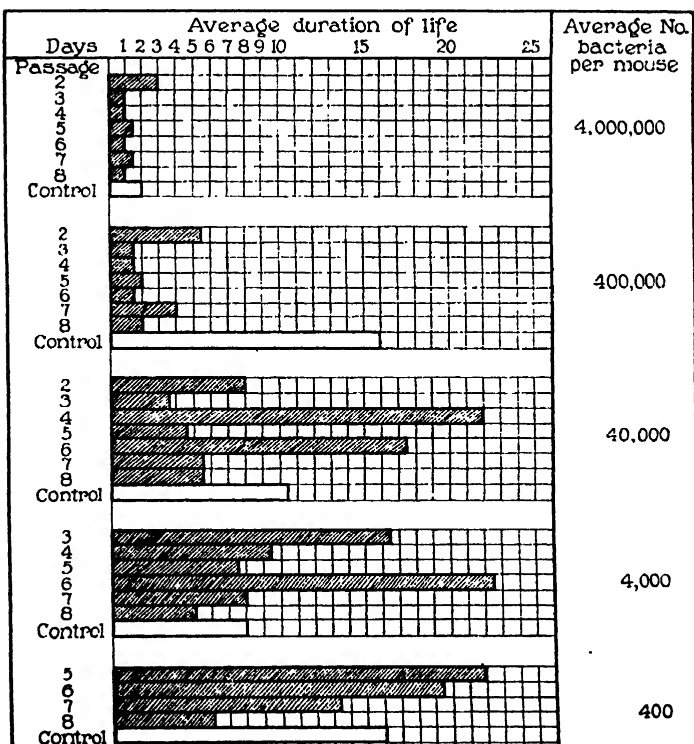
In Text-figs. 1 to 6 the "Average duration of life in days" represents the mean survival time of the two mice used for each dilution. "Average number of bacteria per mouse" represents the mean of the nearest comparable figures for each passage.

strains; and that *Bacillus pestis cavia* and *Bacillus paratyphosus* B show the least virulence.

In brief, then, under the conditions of the experiment, intraperitoneal mouse passage did not affect the virulence of six strains of paratyphoid-enteritidis bacilli.

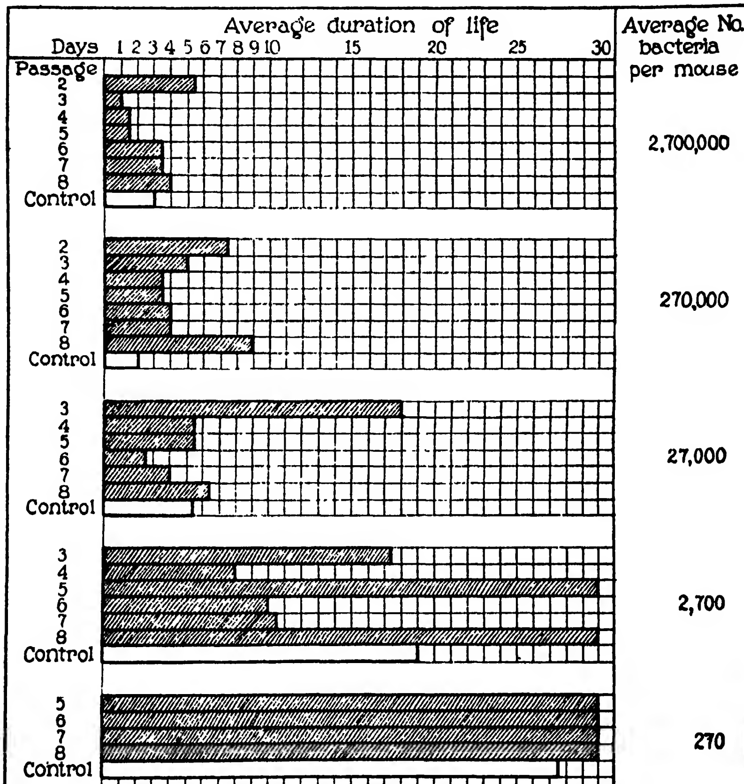


Text-Fig. 2. Intraperitoneal virulence of Strain *B. enteritidis*.



Text-Fig. 3. Intraperitoneal virulence of Strain M. T. II.

Experiment 2 was planned to determine the effect of direct intraperitoneal mouse passage without intermediate artificial cultivation on the virulence of the six strains of paratyphoid-enteritidis bacilli.

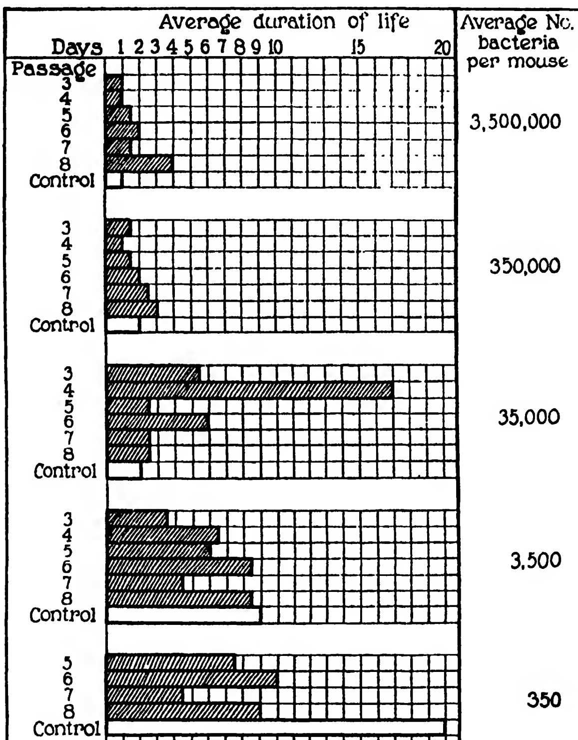


TEXT-FIG. 4. Intraperitoneal virulence of Strain *B. pestis caviæ*.

Method.—Broth cultures from the six stock agar tubes were incubated 6 hours and injected in 1 cc. quantities each intraperitoneally into three mice. 24 hours later, these mice were either dead or dying. From the living mice, 0.5 cc. of blood was taken from the heart and mixed with an equal volume of 2 per cent sodium citrate in 0.85 per cent salt solution. The heart and spleen from the dead mice of the same series were ground in citrate solution in a mortar with sand and combined with the blood suspension.[6] The six resulting emulsions were shaken and diluted

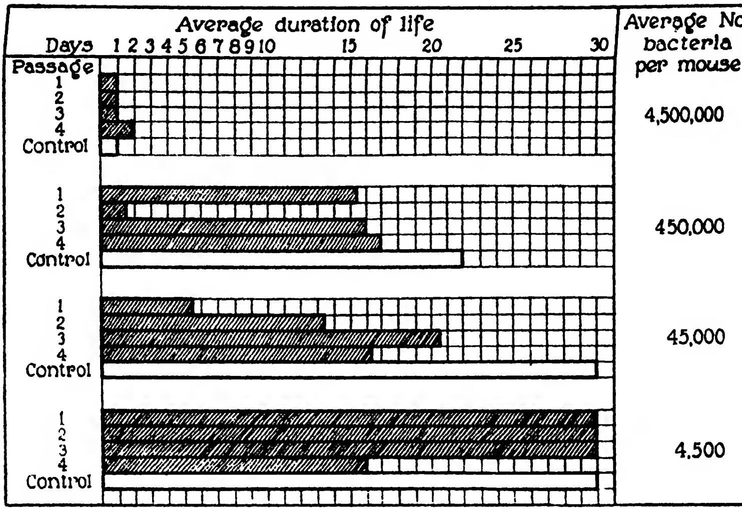
[6] Preliminary test showed that the bacteria taken from the blood during the course of a rapid fatal septicemia do not differ in virulence from those recovered 1 or 2 hours after death from spleen and heart emulsions.

1:100, so that each contained about 3,000,000 organisms per cc. 1 cc. of this dilution of the emulsions was injected intraperitoneally into two mice. 24 hours later, these mice were found in a dying condition. The above procedure was then repeated and continued for five passages. The sixth or final passages were made as follows: dilution 1:10, two mice; 1:100, five mice; 1:1,000, five mice; and 1:10,000, two mice. At this time, control inoculations were made with 18 hour broth cultures from the stock agar tubes in the following dilutions: 1:100, two mice; 1:1,000, five mice; 1:10,000, five mice; and 1:100,000, two mice. The number of colonies per cubic centimeter of each culture was determined by dilution methods so that actual numbers of bacteria per mouse could be used in computing degrees of virulence.

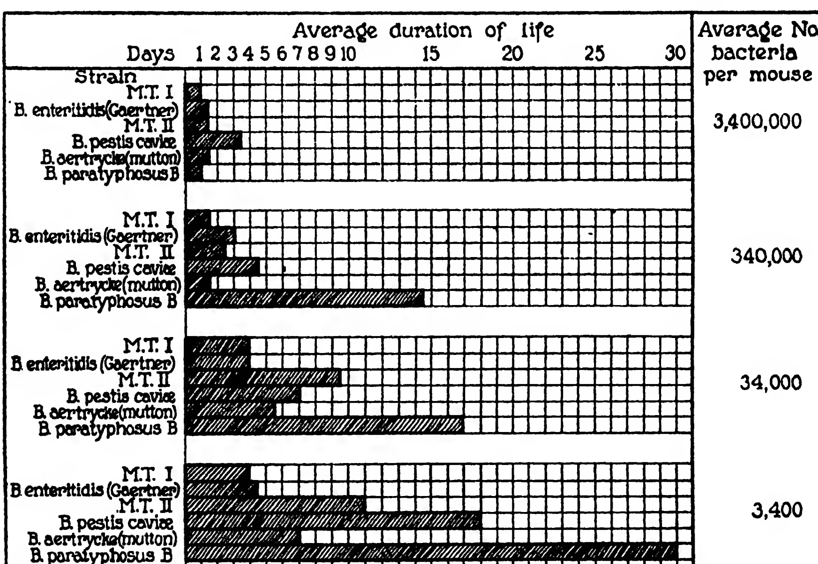


TEXT-FIG. 5. Intraperitoneal virulence of Strain *B. aertrycke* (mutton).

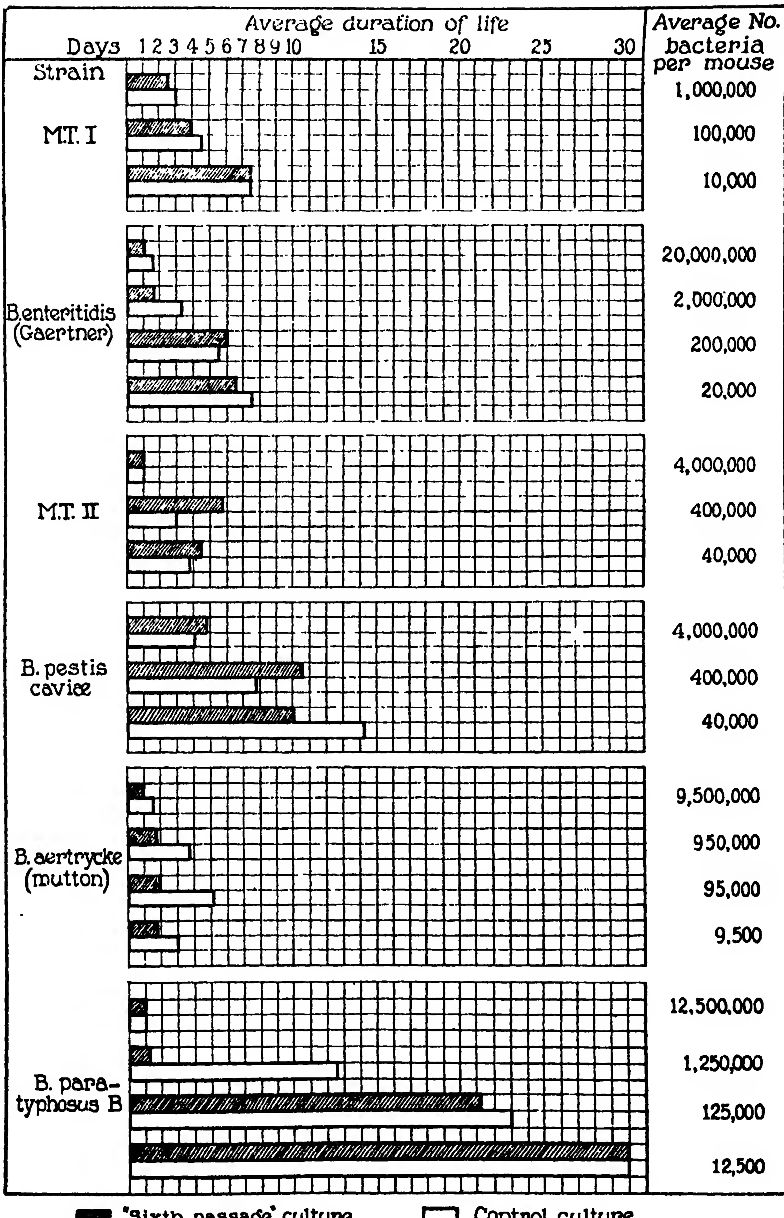
Text-fig. 8, which plots the results of this experiment in the manner described for Text-figs. 1 to 7, shows that direct intraperitoneal mouse passage without intermediate artificial cultivation does not affect the virulence of Strains M. T. I, *Bacillus enteritidis*, M. T. II, and *Bacillus pestis caviæ*. However, there does seem to be a slight



TEXT-FIG. 6. Intraperitoneal virulence of Strain *B. paratyphosus* B.



TEXT-FIG. 7. Intraperitoneal virulences of six paratyphoid-enteritidis strains. "Average duration of life in days" represents the mean survival time of all animals in all passages of the particular strain receiving roughly the same number of bacteria. "Average number of bacteria per mouse" represents the mean of the nearest comparable figures for each of the six series.



TEXT-FIG. 8. Effect of direct passage on the intraperitoneal virulences of six paratyphoid-enteritidis strains.

increase in virulence for each dilution of *Bacillus aertrycke* (mutton) and in one dilution for *Bacillus paratyphosus* B, but when the average duration of life of the passed culture is compared in the various dilutions with the average results shown in Text-fig. 7, the small differences actually become negligible.

From these two experiments we may conclude that direct intraperitoneal passage from mouse to mouse of certain strains of paratyphoid-enteritidis bacilli does not affect materially the basic virulence.

A discussion of the above findings and a further summary will be given in connection with the next paper of the series.[4]

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MICROBIC VIRULENCE AND HOST SUSCEPTIBILITY IN PARATYPHOID-ENTERITIDIS INFECTION OF WHITE MICE. II.

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(Received for publication, March 15, 1923.)

Experimental studies[1] indicate that strains of paratyphoid-enteritidis bacilli differ among themselves in virulence but that for each strain, virulence is relatively a fixed property. It seemed desirable, therefore, to test this point by a series of *per os* passages which would at the same time afford an opportunity to observe infection under more usual conditions and to analyze more accurately the relations existing between the host and the microbic invader.

EXPERIMENTAL.

Experiment 1.—The method adopted has been described elsewhere.[2] Mice weighing 16 to 18 gm. each, taken from the stock breeding room, were segregated two per jar. The six cultures used were obtained from the last intraperitoneal passage reported in the previous paper.[1] Twenty mice were used for each passage of every strain. The dose, 0.5 cc. *per os* of a 16 hour broth culture diluted 1:100, contained approximately 3,000,000 bacteria. The dead mice were autopsied and the cultures obtained from them were identified. From the first mouse to die of each series a blood culture from the heart was made, identified, and after 24 hours incubation, injected into another series of twenty mice. This procedure was repeated with each strain for a varying number of passages and was followed by a control passage with the original, unpassed stock culture. Then it was considered desirable to return to the cultures used for the first *per os* passages and conduct a duplicate series of passages, terminated as before, by a control passage with the original stock culture. Blood cultures were made on the last control series. The technique of this procedure has been described in a previous paper.[2]

Tables I to IV, which illustrate the methods of recording data in each series, show the duration of life and results of blood cultures

[1] Webster, L. T., *J. Exp Med.*, 1923, xxxviii, 33.

[2] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 231.

following injection with *Bacillus enteritidis* and *Bacillus paratyphosus* B respectively. In Text-figs. 1 to 5 are plotted typical curves from each series and in Text-fig. 6 the curves for each strain are compared with one another and with the control Strain M. T. II.

TABLE I.

Per Os Passage. Strain B. enteritidis.

Passage No.	No. of bacteria per mouse.	No. of mice.	Duration of life after injection.	No. of survivors after 60 days.
			days	
1	4,000,000	20	5, 7, 8, 8, 9, 9, 9, 11, 11, 11, 11, 11, 11, 14, 16, 20*, 21, 39*	2
2	3,100,000	20	6, 8, 8, 9, 9, 9, 10, 12, 12, 12, 14, 15, 15, 18, 21, 22, 56*	3
3	3,400,000	20	5, 8, 8, 9, 11, 12, 13, 14, 19, 20, 21, 25, 26, 38, 40, 53, 55	3
4	3,000,000	18	5, 6, 7, 7, 8, 8, 8, 8, 9, 9, 9, 9, 9, 9, 10, 12, 12, 18	0
Control.	3,000,000	20	6, 6, 8, 8, 10, 10, 10, 10, 10, 11, 11, 12, 12, 13, 14, 15, 26	3
1 A	3,500,000	20	6, 8, 8, 8, 8, 8, 9, 9, 9, 9, 9, 10, 10, 10, 12, 15, 16, 28	2
2 A	4,000,000,	20	6, 6, 7, 7, 7, 8, 8, 8, 9, 11, 11, 11, 11, 11, 14, 26, 36	3
Control 2.	5,000,000	20	7, 8, 8, 8, 8, 8, 8, 8, 9, 10, 10, 10, 10, 11, 11, 11, 12, 12, 16, 29	0

* Autopsy cultures sterile.

TABLE II.

Per Os Passage. Strain B. paratyphosus B.

Passage No.	No. of bacteria per mouse.	No. of mice.	Duration of life after injection.	No. of survivors after 60 days.
			days	
1	4,000,000	19	18*, 19, 20*, 21†	15
1 A	5,000,000	20	13, 14†, 14†, 20, 22, 29†, 30	13
2 A	5,000,000	18	8, 14†, 16, 28	16
3 A	3,000,000	20	13, 21	18
Control.	3,000,000	20	6, 18, 19†, 25*	16

* Autopsy cultures sterile.

† Autopsy cultures not identified.

TABLE III.

Blood Cultures from Control 2 Series Mice Inoculated per Os with B. enteritidis on January 31, 1923.

Cage and mouse.	Cultured Feb. 1.	Cultured Feb. 3.	Cultured Feb. 5.	Cultured Feb. 7.	Cultured Feb. 9.	Cultured Feb. 14.	Cultured Feb. 21.	
1 A	–	1 col.	++	+++	Feb. 7. D.			
B	–	–	–	+	1 col.	++	Feb. 15. D.	
2 A	–	–	+++	Feb. 7. D.				
B	–	–	+++	" 7. "				
3 A	–	–	–	+	+++	Feb. 10. D.		
B	–	–	+++	++	Feb. 9. D.			
4 A	–	–	++	++	" 9. "			
B	–	–	+	+++	" 8. "			
5 A	–	–	++	Feb. 7. D.				
B	–	–	+	+	+++	Feb. 10. D.		
6 A	–	–	+++	Feb. 7. D.				
B	–	++	+++	" 6. "				
7 A	–	–	+	–	Feb. 9. D.			
B	–	–	+	–	++	Feb. 11. D.		
8 A	–	–	+++	Feb. 7. D.				
B	–	–	++	++	Feb. 9. D.			
9 A	–	–	+	+	1 col.	–	++	Feb. 28. D.
B	–	–	+++	Feb. 7. D				
10 A	–	–	+	++	+++	Feb. 10. D.		
B	–	–	+	++	+++	" 11. "		

+ indicates 1 to 10 colonies; ++, 10 to 50 colonies; and +++, more than 50 colonies.

The tables and curves indicate that repeated *per os* passage does not alter the virulence of these strains. Strains M. T. I and *Bacillus enteritidis* are shown to have been most virulent and to act similarly.

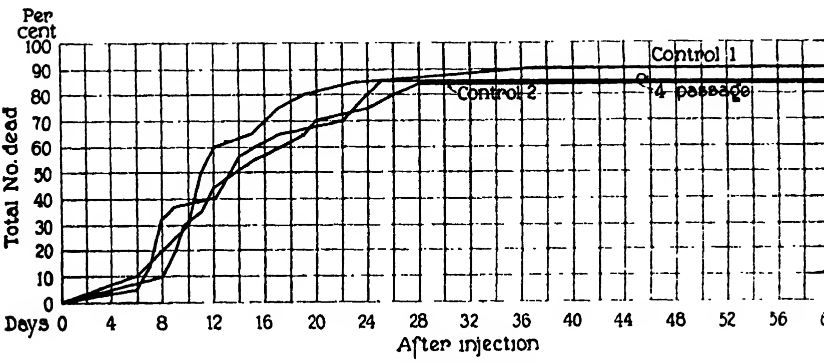
TABLE IV.

Blood Cultures from Control 1 Series Mice Inoculated per Os with B. paratyphosus B on February 8, 1923.

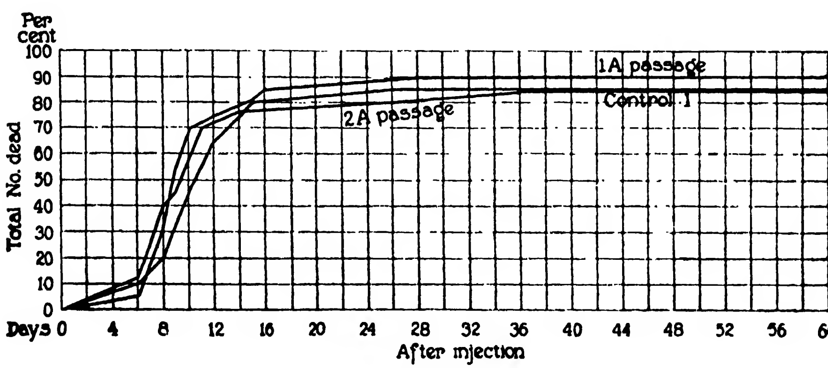
Cage and mouse.	Cultured Feb. 9.	Cultured Feb. 14.	Cultured Feb. 16.	Cultured Feb. 21.	Cultured Feb. 27.	Cultured Mar. 1.	Cultured Mar. 7.	Cultured Mar. 14.	Cultured Mar. 21.
1 A	–	–	–	–	–	–	–	–	–
B	–	–	–	–	–	–	–	–	–
2 A	–	–	–	–	–	–	–	–	–
B	–	–	–	–	–	–	–	–	–
3 A	–	–	–	–	–	–	–	–	–
B	–	–	–	–	–	–	–	–	–
4 A	–	–	–	–	–	–	–	–	–
B	–	–	–	–	–	–	–	–	–
5 A	–	–	–	–	Feb. 26. D.				
B	–	–	–	–	–	–	–	–	–
6 A	–	–	–	–	–	–	–	–	–
B	–	–	–	–	–	–	–	– 1 col. M. T. I.	–
7 A	–	–	–	–	–	–	Mar. 5. D.		
B	–	Feb. 14. D.							
8 A	–	+++	–	–	Feb. 27. Killed by accident.				
B	++	+++	–	1 col.	–	–	–	–	–
9 A	–	–	–	–	–	–	–	–	–
B	–	–	–	–	–	–	–	–	–
10 A	–	–	–	–	–	–	–	–	–
B	–	–	–	–	–	–	–	–	–

+ indicates 1 to 10 colonies; ++, 10 to 50 colonies; and +++, more than 50 colonies.

After *per os* injection positive blood cultures developed promptly and 80 to 90 per cent of the mice died within 3 weeks. Strains M. T. II and *Bacillus aertrycke* (mutton) proved to be less virulent and behaved quite alike. About the same percentage of mice survived, some having shown persistently negative blood cultures, and a few having shown positive cultures and agglutinins. Strains *Bacillus*



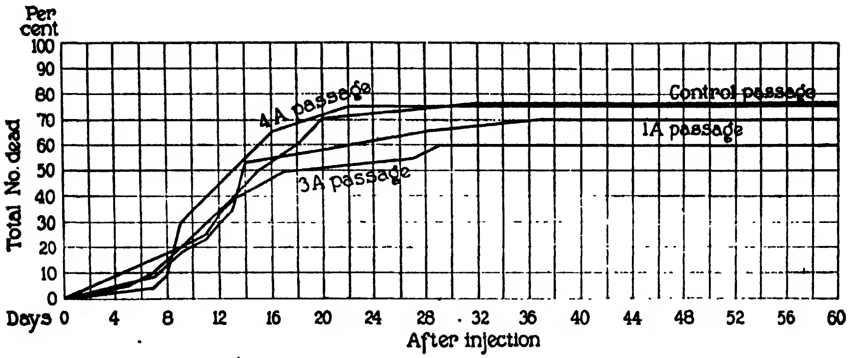
TEXT-FIG. 1. *Per os* passage of Strain M. T. I.



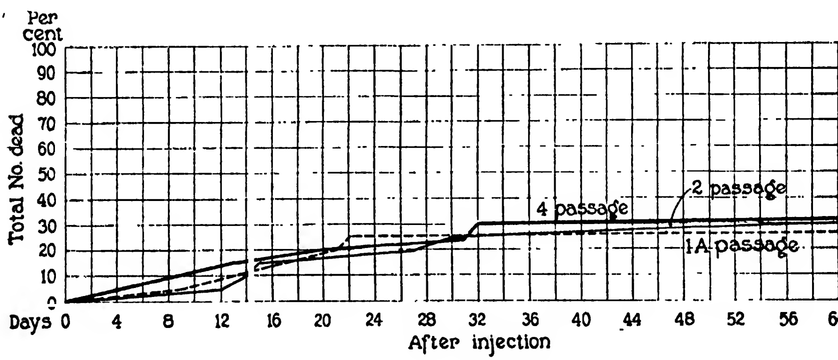
TEXT-FIG. 2. *Per os* passage of Strain *B. enteritidis.*

pestis caviæ and *Bacillus paratyphosus* B affected very few mice in any passage and usually did not invade the blood stream.

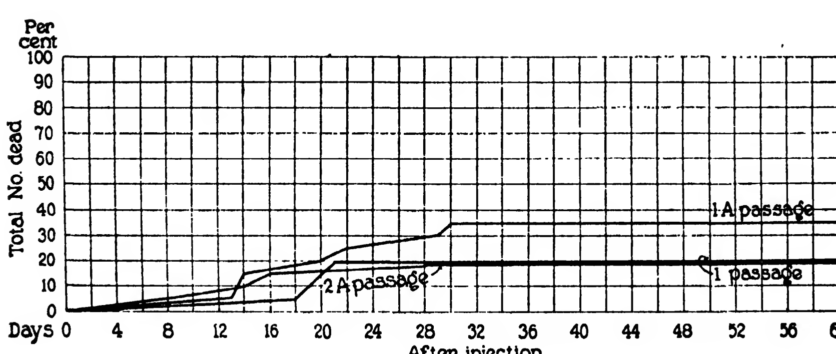
The fixed individual differences in susceptibility to the several pathogenic strains observed among the mice indicated that the reaction was of non-specific nature. In order to test this possibility a simple poisonous chemical compound was administered to a group of mice.



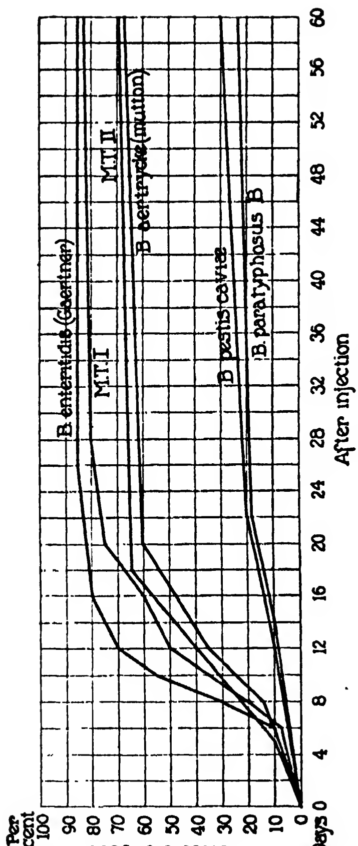
Text-Fig. 3. *Per os* passage of Strain *B. aertrycke* (mutton).



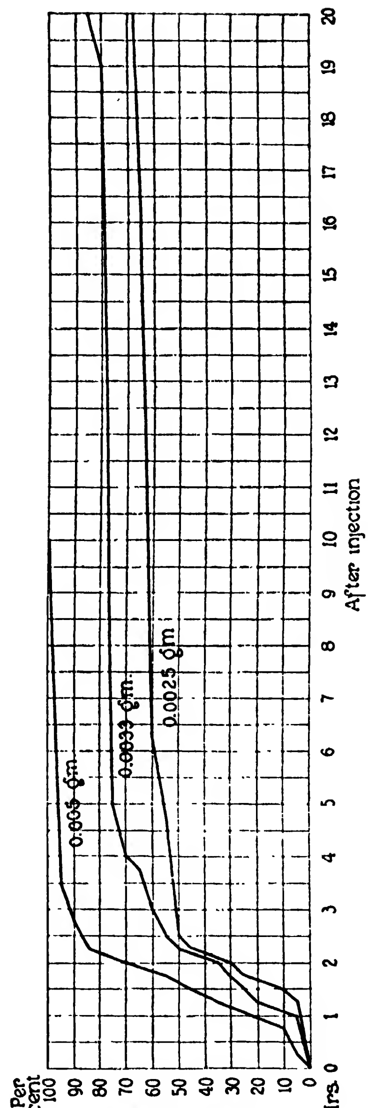
Text-Fig. 4. *Per os* passage of Strain *B. pestis caviæ*.



Text-Fig. 5. *Per os* passage of Strain *B. paratyphosus* B.



TEXT-FIG. 6. Standard mortality curve of six strains of the paratyphoid-enteritidis group.



TEXT-FIG. 7. Mortality curve following *per os* injections of mercury bichloride.

Experiment 2.—From a flask containing 1 gm. of mercury bichloride dissolved in 100 cc. of hot distilled water, subsequent dilutions were made in the usual bacteriological manner more to parallel biological technique than to attain chemical accuracy. The various doses in 0.5 cc. volume were administered *per os* by stomach tube to mice weighing about 16 gm. each.

Test 1: Two mice received 0.05 gm. each, two 0.005 gm., two 0.0005 gm., and two 0.00005 gm. The first two mice were dead in 5 minutes; one receiving 0.005 gm. died in 60 minutes, the other in 18 hours. The remaining mice survived 30 days.

Test 2: Two mice received 0.005 gm. each, two 0.0025 gm., two 0.00125 gm., two 0.0008 gm., and two 0.00061 gm. The 0.005 gm. mice died in 2 hours; the 0.0025 gm. mice were dead in 36 hours; the 0.00125 gm. mice were dead in 72 hours; the remainder survived 30 days.

Test 3: Twenty mice received 0.005 gm. each, twenty 0.0033 gm., and twenty 0.0025 gm. Text-fig. 7 shows the duration of life of these three series.

The mortality curve in Text-fig. 7, following 0.0033 gm. *per os* injection, is similar in form to that resulting from *per os* injection of Strain M. T. I or *Bacillus enteritidis* (Text-figs. 1 and 2), and the curve following the 0.0025 gm. injection resembles the M. T. II and *Bacillus aertrycke* (mutton) curves (Text-fig. 6). The comparison in the latter instance is of special interest since it has been shown previously[3] that the M. T. II curve (Text-fig. 6) may be superimposed upon the Amoss experimental epidemic curves.

Hence it appears that by an adjustment of the dose of mercury bichloride it is possible to secure mortality curves agreeing in form with those resulting from the *per os* injection of living bacteria.

DISCUSSION.

We wish now to correlate our findings with the results of earlier investigations along similar lines. Loeffler[4] and Danysz[6] made the first systematic studies on this group of bacilli in mice, rats, and guinea pigs. While it is true that their main purpose may be regarded as the perfecting of a method for the extermination of rodents, yet they and their successors incidentally or otherwise dealt with such

[3] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 269.

[4] Loeffler, F., *Centr. Bakt.*, 1892, xi, 129; xii, 1.

[5] Mereshkowsky, S. S., *Centr. Bakt., 1te Abt.*, 1895, xvii, 742.

[6] Danysz, J., *Ann. Inst. Pasteur*, 1900, xiv, 193.

questions as the natural spread of the infection in nature and the concurrent fluctuations of virulence among the bacilli.[4–20]

Perhaps it was Danysz who first emphasized the variations of the virulence in order to explain the rise and fall in mortality among the rodents exposed. He was followed by a number of investigators more or less upholding his views on this question, and lately Topley has also adopted this hypothesis as a partial explanation for the epidemic curves obtained in his studies of mouse typhoid.[21] There is, however, no real agreement in the opinions expressed, which range from statements that the virulence fluctuates[6,10,14,15,20] to statements that it remains constant,[13] or that the experimental results are irregular and not interpretable.[8,12,16,17]

We have been impressed with the lack of accuracy in respect to the constancy of dosage, and the small number of animals employed for the experimental tests. Hence our plan included using larger numbers of mice amounting in the end to about 1,400, and controlling the dosage of the bacilli. From the results, which in our case have been generally consistent, we believe that the following may be postulated:

First, the virulence of each of several strains of paratyphoid-enteritidis bacilli is apparently of fixed quantity, for mice at least. It would seem that since the strains used in the present experiments were originally isolated from man and several other animal species

[7] Klein, E., and Williams, H., *Lancet*, 1901, ii, 440.
[8] Kister, J., and Köttgen, P., *Deutsch. med. Woch.*, 1901, xxvii, 275.
[9] Krausz, A., *Deutsch. med. Woch.*, 1901, xxvii, 351.
[10] Bronstein, J., *Deutsch. med. Woch.*, 1901, xxvii, 577.
[11] Abel, R., *Deutsch. med. Woch.*, 1901, xxvii, 869.
[12] Rosenau, M. J., *Bull. Hyg. Lab., U.S.P.H., No. 5*, 1901.
[13] Issatschenko, B., *Centr. Bakt., 1te Abt., Orig.*, 1902, xxxi, 26.
[14] Markl, G., *Centr. Bakt., 1te Abt., Orig.*, 1902, xxxi, 202.
[15] Bahr, L., *Centr. Bakt., 1te Abt., Orig.*, 1905, xxxix, 263.
[16] Trautmann, H., *Z. Hyg. u. Infectionskrankh.*, 1906, liv, 104.
[17] Xylander, *Arb. k. Gsndhtsamte*, 1908, xxviii, 145.
[18] Bainbridge, F. A., *J. Path. and Bact.*, 1909, xiii, 443.
[19] Schern, K., *Arb. k. Gsndhtsamte*, 1909, xxx, 575.
[20] Steffenhagen, K., *Arb. k. Gsndhtsamte*, 1911, xxxvi, 198.
[21] Topley, W. W. C., *Lancet*, 1919, ii, 1; *J. Hyg.*, 1920–21, xix, 350; 1921, xx, 103. Topley, W. W. C., Weir, H. B., and Wilson, G. S., *J. Hyg.*, 1921, xx, 227. Topley, W. W. C., *J. Hyg.*, 1922, xxi, 10, 20.

and cultivated artificially for longer or shorter periods of time, there is good reason for supposing that the virulence of each for mammals in general is relatively a constant. It therefore seems permissible to exclude the factor of fluctuating virulence of the bacilli as explaining the epidemic mortality curve of mouse typhoid.

Next, it is clear from our studies that the different strains of paratyphoid-enteritidis bacilli vary among themselves in virulence for mice. Doubtless similar variations occur for other animals, including man. This inherent pathogenicity becomes, then, an important factor which may determine a severe outbreak of infection or the reverse among a susceptible group of animals. As the strains of high and low virulence may be antigenically identical, the transformation of one into the other cannot be predicated on the basis of bacteriological findings in sick or dead animals. In other words, to determine such transition, it is necessary to employ only accurately planned experiments in which the danger of substitution has been eliminated.

Finally, the experiments have brought out the fact, interesting and perhaps fundamental, that the degree of individual host susceptibility as exhibited by the mortality curve is a general property not restricted to the bacterial infections which we have been studying but inclusive of a chemical intoxication as, for example, with mercury bichloride. It is of importance to know that even when as few as twenty individual mice of a given age and weight, reared alike, are assembled, so small a group will contain individuals highly subject to infection or intoxication, as the case may be, individuals very resistant to the same injurious agencies, and individuals standing between these extremes.

CONCLUSIONS.

1. Six strains of paratyphoid-enteritidis bacilli, isolated from man and animals, differed markedly in virulence for mice.
2. The inherent virulence of each strain for mice was not affected by repeated intraperitoneal and *per os* mouse passage.
3. Individual differences in the susceptibility of the mice to each strain and to graded doses of mercury bichloride were demonstrable. Such variations appear to be of a non-specific nature.

We wish to thank Miss Allen Johnson for assisting with the technical work involved in the experiments reported in this and the preceding paper of the series.[1]

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PRODUCTION OF HETEROGENETIC ANTIBODIES WITH MIXTURES OF THE BINDING PART OF THE ANTIGEN AND PROTEIN.*

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In 1911 Forssman[1] discovered the fact that rabbits injected with suspensions of organs of guinea pigs and a few other animals produce antibodies capable of hemolyzing sheep blood. These hemolysins differ from those which are produced in the usual way by injection of sheep erythrocytes. Because of their origin, they were called heterogenetic antibodies. Later the corresponding antigens (heterogenetic antigens) were found to be present in the tissues of many kinds of animals, but absent in others. Some facts relating to the properties of heterogenetic antigens that have a direct connection with our investigation are the following:[2]

Doerr and Pick[3] detected the resistance of the antigen to alcohol, and Sachs and Georgi[4] made use of alcoholic extracts for their experiments. Friedberger[5] (with Poor, Suto, and Schiff) found the heterogenetic antigen present in the urine of animals to be soluble in alcohol (Doerr and Pick). Sordelli and his coworkers[6] pointed out that the extracts have not only, as known before, the property of combining with the corresponding antibodies but can be flocculated by the immune sera (*cf.* Sachs and Guth[7]). They stated that the extracts are devoid of any power to produce antibodies when injected into rabbits. On the other hand, these authors claimed that heterogenetic antibodies can be produced by injection of the alcohol-

* Sixteenth paper on antigens.

[1] Forssman, J., *Biochem. Z.*, 1911, xxxvii, 78.

[2] For a more complete quotation of the literature, consult the articles mentioned.

[3] Doerr, R., and Pick, R., *Biochem. Z.*, 1913, l, 129.

[4] Sachs, H., and Georgi, W., *Z. Immunitätsforsch.*, *Orig.*, 1914, xxi, 346. *Cf.* Georgi, W., *Arb. k. Inst. exp. Therap. Frankf.*, 1919–20, No. 9, 43.

[5] Friedberger, E., and Suto, K., *Z. Immunitätsforsch.*, *Orig.*, 1919, xxviii, 217.

[6] Sordelli, A., and coworkers, *Rev. inst. bact. Buenos Aires*, 1918, i.

[7] Sachs, H., and Guth, F., *Med. Klin.*, 1920, xvi, 157.

insoluble residue, in spite of its inactivity against the antibodies *in vitro*. In a later review[8] of their work the authors repeated this statement, adding that the antigenic activity of the residue is weak. Landsteiner[9] obtained similar results, but laid stress on the fact that the antigenic power of the residue after alcohol extraction is markedly diminished. Furthermore, it is completely, or almost completely destroyed by treating the material (horse kidney) for a short time with boiling alcohol.

One of the present authors has tried to explain the known facts by the assumption that the heterogenetic antigens consist of two parts, combined with each other and capable of being separated by the action of alcohol. One part is a protein and is necessary for the production of antibodies. The other, which is alcohol-soluble and perhaps a lipoid, contains the specific group but possesses no marked antigenic property and is active as an antigen when combined with proteins. This view was strengthened by experiments[10] which demonstrated that substances of a simple chemical constitution form antigens in combination with proteins and that the corresponding antibodies act specifically on these simple substances. Owing to the lack of a suitable term for such bodies, hitherto but not quite correctly spoken of as antigens, the term haptene was proposed in our previous communication. This term designates, therefore, substances which, while acting strongly and specifically with homologous antibodies, have little or no antigenic properties as compared with the binding power, but become antigens when combined with proteins. It is possible that there exist a number of such substances, as, for example, the alcohol-soluble products studied by K. Meyer[11] and Waelsch, and perhaps the bacterial substances described by Dochez and Avery,[12] Zinsser and Parker,[13] and Heidelberger and Avery.[14] It is still

[8] Sordelli, A., and Fischer, G., *Compt. rend. Soc. biol.*, 1921, lxxxiv, 174.

[9] Landsteiner, K., *Proc. Acad. Sc. Amsterdam*, February, 1921, xxiii, 1166; *Biochem. Z.*, 1921, cxix, 294.

[10] Landsteiner, K., *Biochem. Z.*, 1919, xciii, 106; 1920, civ, 280.

[11] Meyer, K., *Z. Immunitätsforsch., Orig.*, 1910, vii; 1911, ix; xi; 1922, xxxiv.

[12] Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.

[13] Zinsser, H., *J. Exp. Med.*, 1921, xxxiv, 495. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275.

[14] Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 73. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1923, xxxviii, 81.

questionable whether the so called Wassermann antigen has any relation to the present subject (see Seligmann[15]).

The foregoing hypothesis on the nature of the heterogenetic antigen was afterwards independently put forward by Taniguchi[16] and von Gutfeld[17] (see Doerr[18]). In view of the new facts and their interpretation, the existence of nearly related antigens in the tissues of non-related animals appears less surprising than it was previously thought. Such an occurrence would simply depend on the presence of one substance (or several similar substances) of comparatively simple constitution in all these tissues. With regard to the chemical nature of the haptene, according to K. Meyer,[19] it belongs to the group of lecithins and cephalins, while Wernicke and Sordelli[20] designate it as a cerebroside. We believe, however, that the facts put forward up to the present are not yet sufficient to warrant any definite statement as to the purity of the preparations or their precise chemical constitution, especially when one considers the intricacies of the chemical investigation of lipoids.

In order to test the hypothesis above mentioned, we tried to produce antigenic effects by combining the haptenes artificially with proteins—as it were, to synthesize the antigen. The result could not be predicted, as in our previous experiments new antigens were built up from chemical substances which could unite with proteins by means of definite chemical reactions. Such a reaction not being at our disposal in the present investigation, we tried to attain our object by simply mixing the alcoholic extracts with protein-containing solutions. It was thought that in this way a loose (adsorption) compound might be formed.[21] It may be recalled that the natural heterogenetic antigen is also, according to our opinion, a loose compound which can be split up by simple treatment with alcohol.[22]

[15] Seligmann, E., and Pinkus, F., *Z. Immunitätsforsch., Orig.*, 1910, v, 377.

[16] Taniguchi, T., *J. Path. and Bact.*, 1921, xxiv, 253, 254.

[17] von Gutfeld, F., *Z. Immunitätsforsch., Orig.*, 1922, xxxiv, 524.

[18] Doerr, R., *Ergebn. Hyg.*, 1922, v, 168, 130.

[19] Meyer, K., *Biochem. Z.*, 1921, cxxii, 225.

[20] Wernicke, R., and Sordelli, A., *Compt. rend. Soc. biol.*, 1921, No. 3; *Rev. inst. bact. Buenos Aires*, 1919, ii, 281.

[21] It is perhaps possible that the natural hemolysins which are present in the sera employed play a part in the formation of the supposed compounds.

[22] One of the experiments has been briefly reported previously (Landsteiner, K., *Biochem. Z.*, 1921, cxix, 306; *Proc. Acad. Sc. Amsterdam*, 1922, xxiv, 237).

EXPERIMENTAL.

For the injections rabbits were used. To obtain reliable results it was necessary to treat the different series of animals in the same way and at the same time and to use a sufficient number for each experiment. For although the action of heterogenetic antigen is remarkably constant (Doerr) the difference in the reaction of individual animals is great enough to mask the underlying principles when only a small number of animals are studied. Furthermore, in selecting the animals those were excluded in which the serum, prior to the injection, gave a strong or complete hemolysis in a dilution of 1:25 in half an hour under the conditions of the tests. In this way the initial hemolytic activity of the sera was rendered negligible. This method was considered better than to employ rabbits with hemolyzing sera and to calculate the ratio between the initial and the final values, because it is not known whether the production of antibodies in immunized animals is proportional to the initial amount of normal antibodies, other conditions being equal.

Preparation of the Material for Injection.—150 gm. of horse kidney were passed through a mincing machine, sometimes through a sieve. To the pulp were added 750 cc. of 95 per cent alcohol and the suspension was kept at room temperature for 2 days with occasional shaking. The suspension was then filtered and the insoluble residue similarly treated with 450 cc. of 95 per cent alcohol. The first extract was evaporated almost to dryness on a water bath, then dissolved in the filtrate of the second extraction by heat, filtered when hot, and evaporated nearly to dryness. The residue was emulsified in 50 cc. of 0.9 per cent saline.

From this stock solution the injection solutions were prepared by diluting twenty times with 0.9 per cent saline or in the same proportion with diluted serum. The saline emulsions and the mixtures with serum thus contained the same percentage of kidney extract. The sera (human or pig) were diluted eight to ten times with 0.9 per cent saline. To all solutions 0.25 per cent phenol was added. A sufficient quantity of the solutions was made for each series of injections and preserved in the ice box.

Preparation of Antibodies.—The rabbits, fed with vegetables and oats, were injected with 5 cc. of solution each time, intravenously or intraperitoneally, the injections being repeated five to six times at intervals of 7 to 10 days.

Tests for Hemolysis.—The sera of the rabbits were taken 7 days after the last injection. To 0.5 cc. of dilutions of each serum, 0.5 cc. of fresh guinea pig serum, diluted 1:10, was added which itself was not hemolytic under the conditions of the experiment. 1 drop (about 0.05 cc.) of 50 per cent washed sheep erythrocytes was added and the tubes were incubated at 37°C.

I. Injections of Mixtures of Extract and Serum.

Experiment 1 (*Table I*).—Materials injected into various batches of rabbits: (*a*) pig serum 1:10; (*b*) extract of horse kidney; (*c*) the same as (*a*), heated for ½ hour at 80°C.; (*d*) mixture of horse kidney extract and pig serum 1:10; (*e*) the same as (*d*), heated for ½ hour at 80°C. Tests after six intraperitoneal injections. Dilution of the rabbit sera 1:250. Period of incubation 1 hour.

TABLE I.

Material injected.	Pig serum (*a*).				Kidney extract (*b*).				Heated pig serum (*c*).			Mixture of kidney extract and pig serum (*d*).				Mixture of kidney extract and pig serum heated (*e*).			
Rabbit No.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Hemolysis.	0	0	0	F.Tr.	0	0	0	Tr.	0	0	0	C.	M.	Sl.	F.Tr.	Tr.	St.	Sl.	A.C.

In the tables C. indicates complete hemolysis; A.C., almost complete; V.St., very strong; St., strong; M., marked; Sl., slight; Tr., trace; F.Tr., faint trace; and 0, no hemolysis.

The experiment shows a marked difference in the response to the extract and to the mixture of extract and serum. There is no distinct difference between that to the heated and unheated mixtures. No hemolytic power of the sera was found after injections of pig serum in the dilution used.

Experiment 2.—Similar results were obtained after four intraperitoneal injections into three rabbits of alcoholic extract mixed with pig serum diluted 2.5 times. In this instance the mixture was faintly acidified with acetic acid and coagulated by heating for ½ hour to 75–80°C.

Experiment 3 (*Table II*).—Materials injected: (*a*) pig serum diluted 1:8; (*b*) mixture of kidney extract and 1:8 diluted pig serum. Tests after four intravenous injections. Dilution of the sera 1:250. Period of incubation 1 hour.

TABLE II

Material injected.	Pig serum (*a*).				Mixture of kidney extract and pig serum (*b*).		
Rabbit No........................	23	24	25	26	27	28	29
Hemolysis........................	0	0	Tr.	0	0	C.	A.C.

As part of the same experiment four rabbits were injected subcutaneously with the mixture. The sera of these animals never manifested more than a slight hemolytic activity.

Experiment 4 (*Table III*).—Materials injected: (*a*) human serum diluted 1:8; (*b*) extract of horse kidney; (*c*) mixture of horse kidney and human serum diluted 1:8. Tests were made after five intravenous injections. Dilutions of the sera 1:500. Period of incubation 1 hour.

TABLE III.

Material injected..	Human serum (*a*).				
Rabbit No.	34	35	36	37	38
Hemolysis..	0	Tr.	0	0	Tr.

Material injected..	Kidney extract (*b*).											
Rabbit No.	39	40	41	42	43	44	45	46	47	48	49	50
Hemolysis..		Tr.	0	0	0	Sl.	0	Tr.	0	0	A.C.	0

Material injected.	Mixture of kidney extract and human serum (*c*).								
Rabbit No....	52	53	54	55	57	58	59	61	62
Hemolysis....	C.	Sl.	C.	A.C.	Tr.	C.	C.	C.	C.

The dilutions up to which complete hemolysis took place are given in Table IV. According to these results the average titer is about eighteen times higher in the second series than in the first. Moreover, the experiment clearly shows some increase of the hemolytic power in a number of the animals injected with the extract alone. In only one of them is the titer remarkably high (No. 49), but in another experiment, not quoted here, two out of five animals showed a similar increase and one a still higher hemolytic activity. It should be mentioned that in these particular animals coccidiosis was found. It is uncertain whether or not this observation is of any significance.

With regard to the action of the extract alone, an opportunity was afforded in Experiment 4 of comparing in different stages of immunization the sera of rabbits injected with alcoholic extract. The tests were performed at the same time, one set of animals having received two, the other four injections. The dilution of the sera was 1:25, the incubation period 15 minutes. The results are given in Table V.

In addition to the hemolytic activity of the sera, the precipitating power was also tested in some of the experiments in which proteins were injected. It was found that frequently the most active hemolysins and precipitins were developed in the same animals, but this parallelism was not constant.

TABLE IV.

Material injected	Kidney extract (b).											
Rabbit No.	39	40	41	42	43	44	45	46	47	48	49	50
Complete hemolysis up to	1:<25	1:50	1:<25	1:<25	1:25	1:100	1:<25	1:100	1:25	1:<25	1:250	1:<25

Material injected	Mixture of kidney extract and human serum (c).											
Rabbit No.	51	52	53	54	55	56	57	58	59	60	61	62
Complete hemolysis up to	1:1,000	1:1,000	1:100	1:2,000	1:250	1:1,000	1:50	1:2,000	1:1,000	1:2,000	1:1,000	1:1,000

TABLE V.

No. of injections with kidney extract.	Two.						Four.					
Hemolysis	0	Tr.	0	0	Sl.	0	M.	A.C.	St.	0	St.	C.

II. Comparison between Injections of Mixtures and Separate Injections of the Components.

Although a definite increase of the hemolytic activity was not found after injection of either pig or human serum, the possibility had to be borne in mind that by injecting two weakly active substances; *i.e.* extract + serum, instead of a single one a summation of the effects could result. Therefore in the following experiments two series of rabbits were injected. The animals of the first series received 5 cc. of the mixture intravenously as in the preceding experiments. The animals of the second series received 5 cc. of the emulsion of kidney extract into one ear vein and 5 cc. of the diluted serum into the ear vein of the other side. Thus each animal of the two series received the same quantity of both substances, and if the effect had been due simply to an addition, both groups should have reacted in the same degree. This experiment is also related to another point. One might suppose that if a non-antigenic substance is injected into an animal during the period of immunization brought about by an antigen, the former substance would be included, as it were, in the process of immunization. The results obtained, however, do not support such a view.

Experiment 5 (*Table VI*).—Materials injected: pig serum diluted 1:8; and kidney extract. Tests after five intravenous injections. Rabbit sera diluted 1:500. Period of incubation 1 hour.

TABLE VI.

	Separate injections of extract and serum.						
Rabbit No.	63	64	65	66	67	68	69
Hemolysis	0	0	0	V.St.	Sl.	C.	0
Complete hemolysis up to						1:500	

	Mixture.								
Rabbit No.	70	71	72	73	74	75	76	77	78
Hemolysis	C.	V.St.	V.St.	A.C.	A.C.	C.	V.St.	C.	C.
Complete hemolysis up to	1:2,000					1:500		1:1,000	1:500

Experiment 6 (*Table VII*).—Materials injected: human serum diluted 1:8; and kidney extract. In addition to the injections of both components and the mix-

ture, a third series of five rabbits was injected intravenously with diluted human serum alone. Tests after five intravenous injections. Rabbit sera diluted 1:500. Incubation period 15 minutes and 1 hour.

In these two experiments the difference between the two sets of animals in which the same substances were injected in equal quantities but in different ways is very marked. The question as to whether injections of extract and serum made separately are more effective than the injections of extract alone has not yet been studied sufficiently to venture any statement.

TABLE VII.

	Human serum.						
Rabbit No.	79	80	81	82	83		
Hemolysis after 1 hr.	0	0	0	0	0		
	Separate injections of extract and serum.						
Rabbit No.	84	85	86	87	88	89	90
Hemolysis after 15 min.	0	0	0	0	0	M.	0
" " 1 hr.	0	Tr.	Tr.	0	F.Tr.	A.C.	M.
	Mixture.						
Rabbit No.	91	92	93	94	95	96	97
Hemolysis after 15 min.	C.	M.	V.St.	Sl.	A.C.	V.St.	0
" " 1 hr.	C.	A.C.	C.	V.St.	C.	C.	M.
Complete hemolysis up to	1:2,000		1:1,000		1:1,000	1:1,000	

DISCUSSION.

As the preceding experiments show, the antigenic power of heterogenetic antigen destroyed by the action of alcohol can be restored to a considerable extent by admixture of protein solutions such as serum. This effect can be demonstrated relatively easily, as the response of animals to heterogenetic antigen is more regular than with most other antigens, so that when the native antigen is used, antibodies can be obtained from almost every injected rabbit. However, the action of the artificial combination is not equal to that of the native antigen and more intensive treatment is required to produce the same result. Yet in this manner a satisfactory hemolytic titer is frequently obtained.

The related observations thus support the conception that the heterogenetic antigen is composed of two parts, as suggested above.

The supposition that a combination of the alcohol-soluble haptene with the added protein is, in our experiments, the acting substance seems to be the simplest interpretation of the results. The difference in the activity of the sera, when the mixture of haptene and protein is injected as such, or each component separately, demonstrates that some change results from mixing the two substances *in vitro*. The assumption that the effect of the injected mixture is due to some modification other than the formation of a compound seems hardly probable, since a mixture of the haptene with serum also occurs when haptene alone is injected intravenously. In the latter case, however, no marked effect follows. From our point of view this result is comprehensible because homologous proteins are much less adapted to antigenic action than foreign ones. They doubtless can be transformed into antigens[23] even by comparatively slight changes but are always inferior to materials of foreign origin. If doubt remain in the matter it could probably be tested by injections of the heterogenetic haptene mixed with rabbit serum.

With regard to the alcohol-soluble body itself, it is now almost generally admitted[24] that it has no antigenic power. This statement is approximately but not absolutely correct.[25] Observations on a considerable number of repeatedly injected rabbits leave no doubt as to the occurrence of a slight increase in hemolytic power in some of them, even if the few exceptional cases of high titer are ruled out. This finding can be attributed either to real immunization or to an enhanced output of normal antibodies, but the first mentioned explanation seems to be far more likely. There is reason then to believe that after injection of non-protein substances production of antibodies can take place,[26] if to but a slight degree. One can suppose that such an immun-

[23] Pick, R., and Obermayer, F. Landsteiner, K., and Jablons, B., *Z. Immunitätsforsch., Orig.*, 1913–14, xx, 618. Landsteiner, K., and Lampl, H., *Z. Immunitätsforsch., Orig.*, 1917, xxvi, 293.

[24] Sordelli and coworkers, Taniguchi, and von Gutfeld. *Cf.* Meyer, K., *Biochem. Z.*, 1921, cxxii, 225.

[25] *Cf.* Landsteiner, K., *Biochem. Z.*, 1921, cxix, 298, 304.

[26] K. Meyer, Kleinschmidt, Much, and others.

ization is due to the formation of compounds of the haptene with proteins of the rabbit, as in the experiments cited above, and a similar explanation may hold true for the other instances in which antigenic power is ascribed to lipoids, so called. As this view is only hypothetical, it does not enable one to make a sharp distinction between antigenic and non-antigenic substances. But in practise some discrimination is possible because of the wide differences in the activity of the two classes. The immunizing effects ascribed to non-protein substances, as, for example, fats or lipoids, are slight and irregular, and the only substances which can be used effectively for the preparation of antibodies are proteins, if bodies of unknown chemical nature such as toxins are excluded.[27] Consequently it may be assumed that a somewhat different mechanism is concerned in the two instances mentioned. In any case there would appear to be an intimate connection of proteins with the process of antibody production, which is probably not only dependent on the size of the molecules[28] but also on the chemical structure.

The fact that the addition of protein to a substance can transform it into an efficient antigen suggests similar investigation of other substances.[29]

CONCLUSIONS.

1. The alcohol-soluble extract of heterogenetic antigen, which possesses the specific chemical structure of the entire antigen, has a detectable but generally very slight power to increase the amount of heterogenetic antibodies, when injected into rabbits.

2. This substance can be transformed into an efficient antigen by mixing it with protein solutions such as diluted normal serum.

3. Such mixtures are considerably more active than the same substances injected separately. Therefore, the effect of the serum is probably due to the formation *in vitro* of a loose compound between the

[27] See Wells, H. G., Chemical pathology, Philadelphia and London, 2nd edition, 1914.

[28] Landsteiner, K., *Biochem. Z.*, 1919, xciii, 106.

[29] *Cf.* Doerr, R., *Schweiz. med. Woch.*, 1921, ii, 937; Schnabel, A., *Jahresk. ärztl. Fortbild.*, 1920, xi, 15.

alcohol-soluble substance and protein, the compound acting as a complete antigen.

4. It may be supposed that there exists a group of natural antigens which are built up of one specifically reacting part that is almost or entirely devoid of antigenic properties, and another part, a protein, responsible for the immunizing effect.

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ISOLATION OF BACTERIUM PNEUMOSINTES FROM INFLUENZA PATIENTS

During the 1923 Epidemic.*

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The recurrence in January and February, 1923, of an acute respiratory disease resembling the epidemic influenza of 1918–1919, 1920, and 1922 afforded an opportunity to repeat the investigations of Olitsky and Gates[1] on the nasopharyngeal secretions of influenza patients, particularly to attempt the cultivation of *Bacterium pneumosintes* from these secretions. Prior to the onset of the 1923 outbreak, we were engaged in the investigation of the nasopharyngeal secretions from early cases of common colds in man by cultural methods similar to those employed in the studies on influenza. In this connection, it is of interest to record that we failed to obtain cultures of the bacterium from the cases studied.[2] If *Bacterium pneumosintes* could again be cultivated from the nasopharyngeal washings from patients with influenza, the relationship of this microorganism to the latter disease would be strengthened.

In addition to the transmission and cultivation experiments, we shall report also the results of agglutination reactions with *Bacterium pneumosintes*, employing the serums from recovered patients of the 1923 epidemic.

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1. Olitsky, P. K., and Gates, F. L.: Experimental Study of the Nasopharyngeal Secretions from Influenza Patients, J. A. M. A. **74**: 1497 (May 29) 1920; Studies of the Nasopharyngeal Secretions from Influenza Patients, **76**: 640 (March 5) 1921; J. Exper. Med. **33**: 125 (Feb.) 1921; **33**: 713 (June) 1921; **36**: 501 (Nov.) 1922; Science **57**: 159 (Feb.) 1923.

2. To be reported in a forthcoming number of the Journal of Experimental Medicine.

Source of Materials.

A number of infections, some of obscure origin, of the upper respiratory tract occurred concomitantly with the onset and during the course of the epidemic of influenza. These ailments, not unusual for the season of the year, made the selection of cases of true, uncomplicated influenza difficult. Furthermore, we sought patients only in the early hours of the disease. Therefore, of the twenty persons who responded to our call for volunteers, only six were chosen as being representative of the type that prevailed during the pandemic of 1918–1919 and the subsequent recurrences of 1920 and 1922. In the selection of an early case of uncomplicated influenza for study, particular stress was placed on the occurrence of leukopenia affecting the absolute number of mononuclear cells, associated with symptoms and signs considered as typical of an influenzal attack. These consisted of an abrupt onset with chilliness, fever, prostration, headache, and muscular pains, especially in the back and limbs. Among the early symptoms were flush and suffusion of the face, injection of the conjunctivae, soreness of the throat, and a harsh, unproductive cough. The clinical course of Patient 1, a man, is described as illustrative of that of the other five patients studied:

The onset was sudden, with frontal headache, chilly sensations and generalized muscular pains, especially in the back and extremities. There was marked prostration, although the temperature was only 38.9°C. (102°F.). Conjunctivitis was present, accompanied by photophobia. As the illness progressed, a dry and distressing bronchial cough supervened, but physical examination of the chest revealed no lung involvement. Other organs were not affected. After three days, following a profuse perspiration, the temperature fell to normal and the patient recovered. The dry cough and the prostration, however, persisted for several weeks thereafter. Twelve hours after the onset of the first symptoms, a blood count showed the total number of leukocytes to be 3,000, of which 600 were mononuclears. At this time the nasopharynx was washed[1] with 30 c.c. of Ringer's solution, and the washings were retained for study.

The remaining five patients corresponded in practically all the signs and symptoms to the one described, except in the total blood counts, which were, respectively, 4,800, 6,400, 3,800, 7,400 and 4,000 cells. The hour after onset in which the blood count was made and the washings secured from each of these five patients was,

respectively, the forty-eighth, the twenty-fourth, the twenty-fourth, the thirtieth and the sixteenth.

RESULTS OF TRANSMISSION AND CULTIVATION EXPERIMENTS.

A part of the nasopharyngeal washings from each case was filtered, and the filtrate was cultivated on anaerobic blood-agar plates and in the Smith-Noguchi fluid medium by methods already described.[3] The unfiltered material was injected intratracheally in rabbits[1] not only to study pathogenicity, but to aid in the cultivation of *Bacterium pneumosintes*, for it has already been determined[3] that a negative experience in the direct cultivation of this micro-organism without the help of animal passage is not conclusive.

The intratracheal inoculation of the secretions of five of the six patients induced in rabbits the usual clinical and pathologic condition, which consists, in brief, of a nonfatal and transitory leukopenia due to mononuclear depression, associated with a hemorrhagic edema and emphysema of the lungs and characteristic cellular exudate, but without pneumonic consolidation.[1] The nasopharyngeal secretions which failed to induce a reaction in the rabbit were obtained from a patient forty-eight hours after the onset of the first symptoms. In his blood, 7,400 leukocytes per cubic millimeter were found. Thus we were able to confirm, with the secretions from the recent cases of influenza, the results of animal transmissions obtained with nasopharyngeal washings derived from patients during the 1918–1919, the 1920 and the 1922 waves of the epidemic.

Cultivation tests were made with the filtered nasopharyngeal secretions and with the unfiltered suspensions of lung tissue from rabbits inoculated intratracheally with these patients' washings. Of the five persons whose secretions gave positive rabbit transmissions, there was found in the filtered nasopharyngeal washings of one a filter-passing, Gram-negative micro-organism. From the lung tissue itself of a rabbit injected with the material of this patient was also cultivated a similar organism. In two other instances the patients' secretions did not yield this bacterium, but from the respective rabbits' lungs inoculated with the washings, growths of the same micro-organism were obtained.

3. Olitsky, P. K., and Gates, F. L.: J. Exper. Med. 36: 501 (Nov.) 1922.

These filter-passing bacteria, when compared with the strains of *Bacterium pneumosintes* isolated in 1918–1919, 1920 and 1922, were found identical in morphology and cultural characters, and the new strains were specifically agglutinated by immune rabbit serum prepared from the old ones. By the time, however, that the new cultures were purified and grown in sufficient quantity for animal inoculation, a process requiring from three to four months and from eight to twelve generations, tests for pathogenicity in the rabbit failed.

Thus in the 1923 wave of epidemic influenza there were isolated four strains of *Bacterium pneumosintes* from three of the six patients studied in the early hours of the disease. One culture was obtained directly from the filtered nasopharyngeal secretions and three cultures from the lungs of rabbits inoculated intratracheally with the secretions. All the strains agree morphologically, culturally and serologically with the cultures that were obtained in the previous waves of epidemic influenza.

SERUM AGGLUTININS IN PATIENTS WHO RECOVERED.

The agglutination tests were carried out by the method already described.[4] The agglutinogens consisted of two pneumosintes strains; one, Strain 17, obtained from the 1918–1919 wave, and the other, Strain 34, from the 1922 wave of the influenza epidemic. Recent strains were not available when the tests were made.

Of the six patients whose nasopharyngeal secretions were studied and reported in this paper, the serums of four were examined for agglutinins. In one instance no agglutinins were found, even in dilutions of 1:2, seven days after recovery; but agglutinins were demonstrated in dilutions of from 1:2 to 1:10, thirteen days after the illness. In the second case, the agglutination test was positive in from 1:2 to 1:10 dilutions on the forty-seventh day after recovery, the only test performed. The two other serums were wholly negative.

Serums were available from six other recovered patients. We did not observe these cases personally, so that the nasopharyngeal secre-

4. Olitsky, P. K., and Gates, F. L.: Experimental Studies of the Nasopharyngeal Secretions from Influenza Patients, XI, Antibodies in the Blood After Recovery from Epidemic Influenza, J. Exper. Med. 37: 303 (March) 1923.

tions were not collected. However, the physicians in charge of the patients reported them as cases of typical, acute, uncomplicated epidemic influenza. In one patient the record of a blood count is missing; in the remaining five, the total blood counts given were, respectively, 3,200, 3,860, 4,200, 5,200 and 9,200.

Of these six cases, two were examined on the fifth and seventh days of convalescence. Both showed agglutinins in a dilution of serum of 1:2. A second examination, made on the twelfth and thirteenth days after the illness, revealed an increase of the titer in both to 1:8. The serums of the next two patients agglutinated the pneumosintes strains in dilutions of 1:10 on the ninth and tenth days, respectively, of convalescence. In these two instances no other tests were made. The remaining two patients were wholly negative.

To control the agglutinability of the cultures employed, Rabbits A and B, injected intravenously and repeatedly over long periods of time with living *Bacterium pneumosintes*, showed in their serums agglutinins against this micro-organism in dilutions of 1:10 in one case and 1:20 in the other.

To summarize the results of the agglutination tests, it may be said that of the serums from ten recovered patients, four were negative and six were positive. Of the positive cases, three were examined in the early and later stages of the convalescence and showed either slight or no agglutination against *Bacterium pneumosintes* in dilutions of 1:2 from five to seven days after recovery, but an appreciable reaction in dilutions of from 1:8 to 1:10, on the twelfth to the thirteenth day of convalescence. In one instance the agglutinins were demonstrable on the forty-seventh day after the influenzal attack.

The highest agglutinin titer in this series was 1:10. In this connection it is to be noted that in a previous publication[4] it was stated that *Bacterium pneumosintes* is a stable micro-organism which is not easily agglutinated.

SUMMARY.

From the nasopharyngeal secretions of six patients in the early hours of typical, uncomplicated epidemic influenza during the epidemic of January and February, 1923, we observed in five instances

an active agent, pathogenic for rabbits. On culture of the filtered secretions and of the lungs of rabbits inoculated intratracheally with these materials, four new strains of *Bacterium pneumosintes* have been isolated, one directly from the secretions and three from the rabbits' lungs. All the strains have been identified as agreeing with those of the 1918–1919, 1920 and 1922 strains in morphology, cultural properties and serologic reactions.

Agglutinins against *Bacterium pneumosintes* were demonstrated in six of ten patients who had recovered from an influenzal attack.

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OXIDASE REACTION OF VARIOUS GROUPS OF BACTERIA.

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(Received for publication, May 24, 1923.)

The oxidases of both animal and plant tissue have been extensively studied; they are found almost universally in all forms of life and exert undoubtedly an important function in the oxidation in living cells. The constitution of these enzymes and the mechanism of their action are not understood. They have been defined by Portier[1] as "agents found in life which have the power to oxidize certain substances in the presence of oxygen in gaseous or dissolved state." Duclaux[2] states that: "Oxidases are substances which at ordinary temperatures and under physiological conditions carry oxygen rapidly to materials upon which, without the intervention of oxidases, ordinary oxygen would act very slowly." The different oxidases are usually classified on the basis of the substance which is oxidized; that is, a tyrosinase is an oxidase which oxidizes tyrosine; an aldehydase one which oxidizes an aldehyde, etc. Whatever their constitution or mechanism of action, it is understood that oxidases are known to change the so called inactive molecular oxygen into oxygen in the active state, suitable for chemical union, and that without the aid of these agents as an expression of vital activity oxidation takes place very slowly, if at all. Hence, the importance of any study that may furnish a better insight into a chemical reaction so important to all forms of life.

From a survey of the literature it is evident that few investigators have studied or commented on this activity in the case of the ordinary microorganisms, though great attention has been focused upon the oxidases of animal and plant tissue.

[1] Portier, Les oxydases dans la séries animale, leur rôle physiologique, Thèse de Paris, No. 63, Paris, 1897.

[2] Duclaux, E., Traité de microbiologie, Paris, 1899, ii, 565-584.

Schönbein,[3] as early as 1855, made a study of fungi and found positive oxidase activity. Roux,[4] in 1899, demonstrated the existence of an oxidase in a culture of *B. coli*. Yeast was shown to be capable of producing oxidases by Tolomei,[5] Effront,[6] Buchner,[7] Grüss,[8] and Issajew.[9] Dietrich and Liebermeister[10] found this ferment in pustules. Lehmann and Sano,[11] in a study of thirty microbic species, detected the presence of a tyrosinase in three; namely, *B. putidum*, *B. phosphorescens*, and *Actinomyces chromogens*. Kastle's monograph on oxidases[12] provides an excellent bibliographic source and historical presentation of the development of knowledge concerning oxidases.

While estimating the bactericidal action of certain compounds of the triphenylmethane leuco bases, I observed that some of them were changed into their corresponding dyes in the presence of growing pneumococci, a chemical reaction not actuated by individual strains of *Streptococcus hæmolyticus*, *Staphylococcus aureus*, meningococcus, or *Bacillus typhosus*. The oxidative phenomenon was first noted with *p*-aminoleucomalachite green when pneumococci were grown on whole horse blood. Control tubes consisting of whole defibrinated horse blood and the leuco compound remained colorless for a period of 96 hours. With the pneumococcus a deep purple color developed, the shade being that of *p*-aminomalachite green. Obviously, the dye was

[3] Schönbein, C. F., Ueber Ozon und Ozonwirkungen in Pilzen, *Phil. Mag.*, 1856, ii, series 4, 137; Ueber die Selbstbläuung einiger Pilze und das Vorkommen von Sauerstofferregern und Sauerstoffträgern in der Pflanzenwelt, *Verhandl. Naturf. Ges.*, 1855, i, 339; Ueber Ozon und Ozonwirkungen in Pilzen, *J. prakt. Chem.*, 1856, lxvii, series 3, 496.

[4] Roux, Sur une oxydase productrice de pigment, sécrétée par le coli-bacille, *Compt. rend. Acad.*, 1899, cxxviii, 693.

[5] Tolomei, G., Sopra un fermento solubile che si trova nel vino, *Atti Real. Accad. Lincei, Rend. sc. fis., mat. e. nat.*, 1896, v, series 5, 52.

[6] Effront, J., Enzymes et leurs applications, Paris, 1898; English translation by S. C. Prescott, New York, 1902.

[7] Buchner, E., Ueber zellenfrei Gährung, *Ber. chem. Ges.*, 1898, xxxi, 568.

[8] Grüss, J., Über Oxydaseerscheinungen der Hefe, *Woch. Brauerei*, 1901, xviii, 310, 318.

[9] Issajew, W., Ueber die Hefeoxydase, *Z. physiol. Chem.*, 1904, xlii, 132.

[10] Dietrich, A., and Liebermeister, G., Sauerstoffübertragende Körnchen in Milzbrandbacillen, *Centr. Bakt., 1te Abt., Orig.*, 1902, xxxii, 858.

[11] Lehmann, K. B., and Sano, Über das Vorkommen von Oxydationsfermenten bei Bakterien und höheren Pflanzen, *Arch. Hyg.*, 1908, lxvii, 99.

[12] Kastle, J. H., The oxydases, *Bull. Hyg. Lab., U. S. P. H., No. 59*, 1909.

oxidized from its leuco base by the activity of the growing pneumococci.

This observation presented an opportunity for studying the problem of the oxidase activity of bacteria by means of a simple technique. Leucomalachite green had been used for the detection of blood by Adler and Adler[13] and later by Buckmaster[14] in a study of pseudoperoxidase reaction between hemoglobin and its derivatives. The compound used by them was the leucotetramethyldiaminotriphenylmethane, whereas the *p*-aminoleucomalachite green made use of by a simple technique in the present study is the 4, 4,-bis dimethylamino-4-aminotriphenylmethane. We wish to present here, first, a study of the pneumococci in relation to the oxidase activity under various conditions of growth, and, second, an inquiry into the oxidases of other microbic species as determined by this indicator.

Technique.

Solution of the indicator is made by dissolving a weighed portion of the leuco base in dilute hydrochloric acid, which is added drop by drop on the dry chemical. After dissolving, water is added to make a 1:100 concentration and the excess of acid neutralized by dilute sodium hydroxide, enough alkali being used to produce just a slight opalescence. Sterilization can be effected by boiling, or autoclaving under nitrogen, or by means of a Berkefeld filter. For practical purposes, it was found sufficient to boil in an excess of hydrochloric acid, and then neutralize with sterile sodium hydroxide (N) after the solution became cool. The amount of leuco base used in the test represented a final dilution in the media of 1:10,000.

The usual method was to pipette 2 cc. of medium into a 10 mm. tube, add, in turn, 0.1 cc. of a 1:500 concentration of leuco base and then 0.1 cc. of an 8 hour culture of the bacteria in question. The inoculated tubes were incubated for varying lengths of time at 37.5°C. Controls of both medium and indicator were always run.

Conditions Which Influence the Reaction with Pneumococcus.

As stated above, the reaction was first noted when whole defibrinated horse blood was used as medium. Serum was found to give as suitable conditions as whole blood, the one requirement for both being

[13] Adler, O., and Adler, R., *Z. physiol. Chem.*, 1904, xli, 59.

[14] Buckmaster, G. A., The pseudo-peroxydase reaction between hæmoglobin, its derivatives and the leucobase of malachite green, *J. Physiol.*, 1908, xxxvii, p. xi.

that they be fresh. Contrarily, plain broth or meat infusion (plain broth with peptone) and 1 per cent peptone solution, each having a pH of 7.8, prevented the oxidation of the indicator by pneumococci. The difference is striking and perhaps significant. Pneumococci growing in serum cause oxidation of *p*-aminoleucomalachite green, whereas growth, seemingly as abundant on the broth, meat infusion, and peptone media as on serum, produced not the slightest oxidation of the indicator. The reaction is unique in that it appears to require something in fresh animal tissue (fresh serum) as a substrate. Although it is not our purpose to explain the reaction in this communication, the following experiments were performed in quest of a possible reason for the difference in action between media with and without serum.

Experiment 1.—Variation in the concentration of serum, plain broth, and meat infusion from 100 to 10 per cent, in gradients of 10.

Experiment 2.—Variation in the concentration of meat infusion, peptone solution, and plain broth in a quantity of horse serum that rendered possible the oxidation of this indicator.

Experiment 3.—Variation of peptone solution and meat infusion in guinea pig serum.

The results of these experiments are given in Table I. The leuco compound is not oxidized by growing pneumococci in any concentration of the media without serum, while complete oxidation occurs in serum in as high a concentration as 70 per cent. In undiluted serum and in 80 and 90 per cent serum, definite inhibition of the oxidation reaction is manifest. The results in these dilutions indicate the existence of a reducing potential, perhaps an antioxidase, which counterbalances the oxidation by the microorganism. Experiment 2 emphasizes the reciprocal relationship; that is, at a definite concentration of broth and meat infusion media, in a quantity of serum in which a positive reaction would normally be obtained, no oxidation of the indicator is observed. It might be thought that the meat infusion, broth, and peptone media have a destructive action on the oxidizing enzymes of the serum; indeed, this mechanism might be explanatory of the difference between the action of these media and serum, were it not for the fact that undiluted serum inhibits or prevents the reaction in a similar way.

By chance, guinea pig serum was used in one of the experiments instead of horse serum, and the impression was gained that oxidation proceeded much more readily in guinea pig serum than in horse serum. To study the question further, Experiment 3 was carried out. The

TABLE I.

Influence of Different Media on Oxidase Production by Pneumococcus.

Medium.	Diluent.	Percentage of dilution.										
		Medium.										
		100	90	80	70	60	50	40	30	20	10	0
		Diluent.										
		0	10	20	30	40	50	60	70	80	90	100
Meat infusion (pH 7.8).	Buffer salt (pH 7.8).	0	0	0	0	0	0	0	0	0	0	0
Plain broth (pH 7.8).	" " (pH 7.8).	0	0	0	0	0	0	0	0	0	0	0
Fresh horse serum.	" " (pH 7.8).	1	1	2	4	4	4	4	4	4	4	0
20 per cent fresh horse serum.	20 per cent meat infusion.	4	3	2	2	1	1	Tr.	0	0	0	0
20 " " " " "	20 per cent of 1 per cent peptone solution.	4	3	2	1	0	0	0	0	0	0	0
20 " " " " "	20 per cent plain broth.	4	3	2	1	0	0	0	0	0	0	0
20 " " " guinea pig serum.	50 per cent meat infusion.	4	4	4	4	4	4	4	4	3	2	0
20 per cent fresh guinea pig serum.	50 per cent of 1 per cent peptone solution.	4	4	4	3	2	1	Tr.	0	0	0	0

The figures 1, 2, 3, and 4 represent respectively 25, 50, 75, and 100 per cent oxidation as judged by the intensity of the color of the indicator. 0 means no visible oxidation.

All tubes contained 2 cc. of medium and 0.2 cc. of 1:500 *p*-aminoleucomalachite green.

Observation made after 48 hour period of growth.

results show definitely that guinea pig serum possesses the essential elements for the oxidation of the indicator by pneumococci to a greater degree than horse serum. If undiluted meat infusion is used as a basis for comparison it is seen that a 1 per cent mixture of meat infusion

with a constant amount of horse serum inhibits the reaction; whereas, 40 per cent of meat infusion is necessary to cause the same inhibition in guinea pig serum. Because of the result of the last experiment, sera from seven different animal species, under as nearly the same conditions as practicable, were studied. The experiment with each specimen was repeated three times with sera of rat, guinea pig, rabbit, horse, man, cat, and chicken, with the following results. In order of suitability for the oxidation reaction, the general grouping was: rat, guinea pig, and rabbit best; horse next best; man, cat, and chicken least suitable. There exists the possibility that the variation in the sera from different animal species may be due to the presence of hemoglobin, though reasonable care was exercised in securing the specimens of sera. The animals, with the exception of the horse, were bled from the heart by means of a syringe with a new needle, both of which had been washed out with normal physiological salt solution prior to use.

In addition, it has been noted that hemoglobin influences the reaction; a small amount is stimulatory, a larger quantity inhibitory. Oxygen is essential for the reaction itself, no change taking place under anaerobic conditions. However, if air is admitted to an 8 hour culture which has been grown anaerobically in suitable medium, oxidation takes place almost immediately. H ion concentration between the limits of pH 6 and 7.8 increases the rate of oxidation in direct ratio to the H ion concentration,—the more acid the media, the more rapid the reaction. Virulence of the pneumococci does not seem to cause any change in the degree or rate of oxidation of the indicator. During the course of another investigation, the virulence of a strain of pneumococcus was so altered that the minimum lethal dose of various cultures for mice was as follows: 2, 1,800, 46,000, 120,000, and 3,000,000 organisms. The cultures were tested in varying amounts of meat infusion in a constant quantity of guinea pig serum. No difference in the reaction was discernible. It was also found that heating fresh serum for 30 minutes at 55°C. made it more suitable for the reaction, in part destroying the inhibiting power of undiluted serum. A concentration of 1 per cent dextrose in serum was found to increase the rate of oxidation by pneumococci.

Reaction with Different Microbic Species.

In applying the test to other species of microorganisms, we have made use of conditions for oxidase production which were optimum for pneumococci; namely, 10 per cent fresh horse serum, 10 per cent fresh horse serum with the addition of 1 per cent dextrose, and 10 per cent fresh guinea pig serum. The work represented in the tables was done as a series of complete experiments. A sample of serum and indicator which was to be employed for the experiment was tested prior to its use to establish its suitability as a substrate for oxidase formation by growing pneumococci.

TABLE II.

Failure of the Oxidase Reaction.
Miscellaneous Group of Bacteria.

Organism.	No. of strains.	Medium pH 7.8.			
		10 per cent horse serum.	10 per cent horse serum with 1 per cent dextrose.	10 per cent guinea pig serum.	Plain broth.
Staphylococcus aureus.	6	0	0	0	0
" *albus.*	4	0	0	0	0
Meningococcus.	14	0	0	0	0
B. typhosus.	3	0	0	0	0
" *paratyphosus* A.	2	0	0	0	0
" " B.	3	0	0	0	0
" *coli communis.*	5	0	0	0	0
" " *communior.*	2	0	0	0	0
" *dysenteriæ* Flexner.	2	0	0	0	0
" " Shiga.	3	0	0	0	0
" *friedländeri.*	1	0	0	0	0
" *lactis aerogenes.*	2	0	0	0	0
" *alkaligenes.*	1	0	0	0	0
" *pyocyaneus.*	3	0	0	0	0
" *proteus vulgaris.*	1	0	0	0	0
" *cereus.*	1	0	0	0	0
" *subtilis.*	1	0	0	0	0
" *anthracoides.*	1	0	0	0	0
" *violaceus.*	1	0	0	0	0
Spirillum choleræ asiaticæ.	1	0	0	0	0

1. Miscellaneous Group.—Table II represents the results of the study of twenty different microbic species comprising 57 strains.[15] It is at once apparent that, under the same conditions in which the pneumococcus causes complete oxidation of the aminoleucomalachite green, no oxidation of the indicator occurs with the other members of the microbic species tested.

TABLE III.

Positive Oxidase Reaction.
Pneumococcus of Human Origin.

Pneumococcus.	10 per cent horse serum.		10 per cent horse serum with 1 per cent dextrose.		10 per cent fresh guinea pig serum.	Plain broth.	1 per cent dextrose broth.
	Period of observation.						
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	72 hrs.
F 55	4	4	4	4	4	0	0
A 66	4	4	4	4	4	0	0
J 36	4	4	4	4	4	0	0
F 104	2	4	2	4	4	0	0
Lister G	2	4	2	4	4	0	0
Ca 81	4	4	4	4	4	0	0
Lister F	4	4	4	4	4	0	0
Melford	Tr.	4	2	4	4	0	0
V 1 16	4	4	4	4	4	0	0
Neufeld	4	4	4	4	4	0	0
169	Tr.	4	1	4	4	0	0
168	4	4	4	4	4	0	0
D 40	4	4	4	4	4	0	0
F 208	3	4	3	4	4	0	0
Jones	2	4	2	4	4	0	0
Lister A	2	4	3	4	4	0	0
"	Tr.	4	3	4	3	0	0

2. Pneumococcus.—As with the other bacteria, these strains were picked from single colonies and grown on serum broth and were stained to determine purity prior to use in the test.[16]

[15] With the exception of the meningococcus, one strain each of the organisms given in the table were old stock cultures kindly furnished by Dr. Stanhope Bayne-Jones. The meningococci were stock strains from The Rockefeller Institute. The remaining cultures had been recently isolated from different sources.

[16] We are indebted to Dr. O. T. Avery for the excellent collection of strains of both pneumococci and streptococci here studied. The latter organisms comprised a large portion of those described by Avery and Cullen (Avery, O. T., and Cullen, G. E., The use of the final hydrogen ion concentration in differentiation of *Streptococcus hæmolyticus* of human and bovine types, *J. Exp. Med.*, 1919, xxix, 215).

In contradistinction to the results obtained with the organisms of the above miscellaneous group, nine out of seventeen strains of pneumococci (Table III), irrespective of type, grown on 10 per cent horse serum, with or without dextrose, completely oxidize the leuco compound in a 24 hour period, all of them in 72 hours. The difference between the influence of horse serum and guinea pig serum is here emphasized. Whereas in 24 hours on the horse serum, nine out of

TABLE IV.

Positive Oxidase Reaction.

Streptococcus viridans of Human Origin.

Streptococcus viridans.	10 per cent horse serum pH 7.8.		10 per cent horse serum pH 7.8, with 1 per cent dextrose.		10 per cent guinea pig serum pH 7.8.	
	Period of observation.					
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.
B/26	Tr.	4	0	4	2	4
A/148	"	4	0	4	4	4
A/179	"	4	1	4	4	4
A/141	"	4	0	4	3	4
A/135	"	4	Tr.	4	4	4
380	"	4	"	4	4	4
A/4	"	4	"	4	4	4
R/0	"	4	"	4	4	4
B/38	"	4	"	4	2	4
B/39	1	4	1	4	2	4
B/26	1	4	1	4	4	4

seventeen strains produce a No. 4 reaction (100 per cent), in this same length of time with guinea pig serum, complete oxidation occurred in sixteen out of seventeen, the remaining one producing a reaction but slightly less (No. 3, or 75 per cent).

3. Streptococcus viridans.—*Streptococcus viridans*, like pneumococci, gave a positive reaction with this indicator, but seemingly at a lower rate (Table IV). Growth for 24 hours is marked by only a slight oxidation, which, however, becomes complete in 72 hours. Again, guinea pig serum enhances the reaction, and to such a degree that in the serum of this animal the demarcation between the strains of *Streptococcus viridans* and pneumococci becomes greatly obscured.

TABLE V.

Positive Oxidase Reaction.
Hemolytic Streptococci of Human Origin.

Streptococcus.	10 per cent horse serum pH 7.8, without dextrose.		10 per cent horse serum pH 7.8, with 1 per cent dextrose.		10 per cent guinea pig serum pH 7.8.
	Period of observation.				
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.
79	Tr.	1	Tr.	4	1
47	"	3	"	4	1
267	0	1	0	2	1
E 7	0	2	0	4	1
43	0	2	0	4	2
A 49	0	4	0	4	3
E 208	0	1	0	4	1
E 223	0	1	0	4	1
239	0	1	0	4	3
E 200	0	2	0	4	2
151	0	1	0	4	1
95	0	0	0	0	1
66	0	2	0	4	1
38	2	2	2	4	2
10	0	2	0	4	2
Boston	0	1	0	4	2
149	0	1	0	4	1
46	0	2	0	4	1
56	0	2	0	4	1
E 238	0	2	0	4	1
8	0	2	0	4	1
27	0	1	0	4	1
92	0	2	0	4	1
89	Tr.	3	1	4	1
25	0	1	0	4	1
S 4	Tr.	2	Tr.	4	1
E 232	0	2	0	2	2
M 29	0	2	0	4	1
M 48	0	1	0	4	1
143	0	0	0	1	3
16	0	1	0	4	1
S 84	0	1	0	4	2
134	0	0	0	0	1
286	0	2	0	4	1
148	0	0	0	4	2
83	0	1	0	4	1
11	0	1	0	4	1
50	0	2	0	4	3
E 265	0	1	0	4	1

TABLE V—*Concluded.*

Streptococcus.	10 per cent horse serum pH 7.8, without dextrose.		10 per cent horse serum pH 7.8, with 1 per cent dextrose.		10 per cent guinea pig serum pH 7.8.
	Period of observation.				
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.
72	0	2	0	4	1
E 256	0	2	0	4	2
271	0	2	0	2	1
134	0	2	0	4	2
59	0	2	0	2	1
44	0	2	0	4	1
C 67	0	2	0	4	1
2	Tr.	2	0	4	1
39	0	0	0	0	1
15	Tr.	1	0	4	2
140	0	1	0	4	2
48	0	1	0	4	1
C 251	Tr.	Tr.	0	1	1
29	0	1	0	4	1
144	0	1	0	4	2
150	0	3	0	4	1
S 6	0	3	0	4	2
S 71	0	3	0	4	3
S 93	0	3	0	4	Tr.
S 128	0	1	0	4	1
S 116	0	Tr.	0	4	1
S 97	0	1	0	4	1
S 85	0	2	0	4	1
S 35	Tr.	1	0	4	1
S 14	"	1	0	4	2
118	"	1	0	4	1
S 24	0	2	0	4	2
S 1	0	0	0	4	1
E 236	0	2	0	4	1
S 108	0	2	0	4	1
96	0	2	0	4	1
S 148	0	Tr.	0	4	1
209	Tr.	1	0	4	2
F 1	0	Tr.	0	4	1

4. *Hemolytic Streptococci of Human Origin.*—The study by this test of 73 strains of hemolytic streptococci of human origin demonstrated a difference from pneumococcus and perhaps in a slight degree from *Streptococcus viridans*; that is, with horse serum media, with or

without dextrose, the reaction was negative in a 24 hour period save in the case of one strain (No. 38); on horse serum without dextrose, the reaction after 72 hours ranged from nil with the majority of strains to a complete oxidation with one (No. A 49). But in horse serum to which dextrose had been added, all but ten strains (Nos. 267, 95, E 232, 134, 39, 59, 271, 143, and C 251, Table V) gave a No. 4 (100 per cent) reaction in 72 hours. *A priori* it seems likely that the influence of the dextrose with the serum is one of H ion concentration. That may be true with the hemolytic streptococci of human origin, but as shown above in the miscellaneous group (Table II), in spite of the low final H ion concentration of the media to which dextrose had been added, no oxidation of the indicator resulted. Although H ion concentration influences the rate of oxidation of *p*-aminoleucomalachite green by certain species of organisms that possess this oxidative faculty, in no case in which the reaction was negative for 72 hours was it possible to bring about the oxidation of the indicator by changing the H ion concentration of the serum. The specific oxidizing power seems to be an inherent characteristic of certain species of organisms. The degree of oxidation in guinea pig serum indicates that the hemolytic streptococcus of human origin possesses lower oxidizing activity than either *Streptococcus viridans* or pneumococcus.

5. Hemolytic Streptococci of Milk Origin.—With this group of streptococci the reaction is much less pronounced than with the bacteria studied above (Paragraphs 2 to 4) and the type of reaction approaches closely that of miscellaneous Group 1. All, excepting four strains, reacted either negatively or but slightly positively. One of these (No. M 53) was isolated by Jones[17] from the milk of a cow showing no evidence of mastitis and was considered by him as perhaps of human origin. The uniformity of the reaction with guinea pig serum demonstrates clearly that while these strains are able to oxidize the indicator, the reaction is less pronounced than with the other species studied (Table VI).

6. Hemolytic Streptococci of Cheese Origin.—It is interesting and perhaps significant that the members of this group react in a fashion

[17] Jones, F. S., Studies in bovine mastitis. II. The relation of hemolytic streptococci to udder infections, *J. Exp. Med.*, 1918, xxviii, 253.

TABLE VI.

Variations in the Oxidase Reaction.
Hemolytic Streptococci from Milk.

Streptococci from milk.	10 per cent horse serum pH 7.8.		10 per cent horse serum pH 7.8, with 1 per cent dextrose.		10 per cent guinea pig serum pH 7.8.
	Period of observation.				
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.
Indian Head.	0	0	0	0	0
Eagle Lunch.	Tr.	0	1	1	3
Munster Brand.	"	0	0	1	4
Philadelphia No. 1.	0	0	0	Tr.	1
" Cream.	Tr.	0	0	0	Tr.
Walker-Gordon.	0	0	0	1	1
Ancre Brand.	0	0	0	0	2
Philadelphia 0.	0	0	0	0	1
Eagle Brand.	0	0	0	0	2
Sheffield (C).	0	0	0	0	3
" (F).	Tr.	Tr.	0	1	1
Crawford Farms.	0	0	0	0	3
Sheffield (E).	0	1	0	4	3
M 3	0	0	0	0	2
M 26	0	2	0	4	2
M 65	0	2	0	2	1
M 43	1	Tr.	1	0	2
M 95	Tr.	0	0	1	1
M 53	0	4	0	4	2

TABLE VII.

Variations in the Oxidase Reaction.
Hemolytic Streptococci from Cheese.

Streptococci from cheese.	10 per cent horse serum pH 7.8.		10 per cent horse serum pH 7.8, with 1 per cent dextrose.		10 per cent guinea pig serum pH 7.8.
	Period of observation.				
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.
Ch 3	Tr.	0	0	1	3
Ch 12	"	0	1	0	3
Ch 2	"	0	Tr.	Tr.	3
Ch 14	"	0	0	0	3
Ch 10	"	0	0	1	2
Ch 15	0	0	0	0	3
Ch 22	0	0	0	0	1
Ch 11	0	0	0	0	3

similar to those of milk origin. The limited number of strains studied does not warrant the conclusion that the strains from milk and cheese origin can be characterized as identical. However, the streptococci from these sources are differentiated in the present study both from streptococci of human origin and streptococci of udder of cow and mastitis (Table VII).

TABLE VIII.

Variations in the Oxidase Reaction.
Hemolytic Streptococci from Cow's Udder and Mastitis.

Organism.	10 per cent horse serum pH 7.8.		10 per cent horse serum pH 7.8, with 1 per cent dextrose.		10 per cent guinea pig serum.
	Period of observation.				
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.
Streptococci from udder.					
V 3	Tr.	1	0	4	1
V 7 5/6	0	2	0	4	1
V 9	1	4	0	4	3
V 5	0	2	0	4	2
V 2	0	2	0	4	2
V 1	0	2	0	3	1
V 8	0	2	0	4	1
V 20	0	0	0	4	1
Streptococci from mastitis.					
C 69	0	0	0	4	2
C 53	0	2	0	4	1
C 57	0	2	0	4	1
C 67	0	2	0	4	1
C 42	0	2	0	4	1

7. *Hemolytic Streptococci from Udder and Mastitis.*—Whatever the truth concerning the origin of the hemolytic streptococci isolated from milk and cheese, the test is not differential for organisms from human or bovine sources. Strains obtained from the udder or from mastitis of cows give manifestly the same reaction as those of human origin (Table VIII).

DISCUSSION.

It has been shown that certain bacterial species in common with both plant and animal tissue are capable of producing an oxidase as

determined by the oxidation of *p*-aminoleucomalachite green. We are not prepared to state that the effect on this indicator of pneumococci is characteristically that of an oxidase. However, if we hold to Portier's definition of an oxidase,—an enzyme capable of changing gaseous oxygen into active oxygen,—we may assume that the oxidation of *p*-aminoleucomalachite green under the conditions reported in this paper is an indication of the presence of such an enzyme.

In the present report the attempt at the quantitative estimation of the degree of oxidase activity, as judged by the intensity of the color of the oxidized leuco base, has been essentially an approximation. However, sufficient work has since been done with a quantitative colorimeter technique to establish this approximation as a fair index of oxidase activity. In addition, despite considerable difficulty with electrodes, the potential in a typical oxidation and reduction set-up has revealed the possibility of using the method in a more exact study. The feasibility of the measurement of oxidation-reduction potential in biological fluids has been shown by Potter[18] in the case of viable yeast, and Gillespie[19] in the case of bacteria in water-logged soils. More recently, Clark[20] states that he has confirmed Gillespie's work in the main and has been able to develop a system of oxidation and reduction indicators which may be used colorimetrically to determine oxidation-reduction potentials.

The indicator described here separates a relatively small group of microbic species into a group distinct from other known pathogens. The line of demarcation is definite; that is, under the most favorable conditions found by us under which oxidation of the indicator by pneumococci occurs (dextrose in serum) there is not the slightest trace of oxidation discernible with any of the strains studied, except the pneumococcus, *Streptococcus viridans*, and *Streptococcus hæmolyticus*. It is not believed that the other bacteria are incapable of oxidation of this character, but rather that the oxidation potential produced by the

[18] Potter, M. C., Rate of fermentation as measured by difference of potential, *Proc. Univ. Durham Phil. Soc.*, 1912, iv, 230.

[19] Gillespie, L. J., Reduction potentials of bacterial cultures and of water-logged soils, *Soil Science*, 1920, ix, 199.

[20] Clark, W. M., Studies on oxidation-reduction. I. Introduction, *Pub. Health Rep., U. S. P. H.*, 1923, xxxviii, 443.

growing organism is not effective for the indicator in question. This view is strengthened by the fact that there is considerable variation in the degree of oxidation among the species giving a positive reaction with this leuco base. Pneumococcus gives the most intense reaction, *Streptococcus viridans* next, and *Streptococcus hæmolyticus* follows, the result varying in part with the source of the organism. The findings with the hemolytic streptococci of human origin and cow's udder and mastitis are not only most pronounced relatively speaking but are indistinguishable from one another, while the organisms from milk and cheese though apparently similar to those just mentioned are of lower oxidative power. We consider these results as a preliminary study. Further work has been confirmatory, but an insufficient number of strains of organisms has been studied to make generalization advisable.

SUMMARY AND CONCLUSIONS.

1. A simple technique is described for studying the oxidase action of bacteria by means of the oxidation of *p*-aminoleucomalachite green.

2. It is shown that pneumococci under aerobic conditions produced an oxidase when grown on suitable medium. The sera of any of seven different animal species constitute such a medium, the degree of oxidation by the pneumococcus depending upon the animal from which the serum was taken—rat, guinea pig, rabbit, horse, man, cat, and chicken in order of diminishing suitability.

3. Conditions favoring the oxidation of *p*-aminoleucomalachite green by a single strain of pneumococci are: the presence of a slight amount of hemoglobin, dextrose, H ion concentration on the acid side, and heating of fresh serum for 30 minutes at 56°C. Conditions preventing the oxidation are: sterilized meat infusion, 1 per cent peptone, plain broth, a high concentration of hemoglobin, and absence of oxygen. In a quantitative fashion, meat infusion, 1 per cent peptone, and plain broth interfere with the suitability of serum as a substratum of oxidase production by the pneumococcus.

4. Twenty-three microbic species were studied with reference to oxidative power. They were grown upon 10 per cent horse serum, with and without dextrose, upon 10 per cent guinea pig serum, and upon plain broth. Only three of the twenty-three gave evidence of oxida-

tive power as tested by *p*-aminoleucomalachite green; namely, the pneumococcus, *Streptococcus viridans*, and *Streptococcus hæmolyticus*. Among the strains, of these three pneumococci gave the most intense reaction, after which *Streptococcus viridans* and *Streptococcus hæmolyticus* follow in the order named, but with a noticeable variation among the different strains of *Streptococcus hæmolyticus*.

5. Hemolytic streptococci of human and bovine origin were studied. The only variation in the type of reaction was manifested by the streptococci of milk and cheese origin. Strains from these sources showed definitely the least oxidase activity. Streptococci from mastitis and cow's udder were indistinguishable by the test from the hemolytic streptococci of human origin.

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CONDITIONS DETERMINING THE TRANSPLANTABILITY OF TISSUES IN THE BRAIN.

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PLATES 13 TO 16.

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The study of the transplantability of animal tumors is largely responsible for the view that it is impossible among warm blooded animals to graft tissues from one species to another, and that even grafting within a species has definite limitations. Leo Loeb,[1] among others, has brought forward evidence indicating that the conditions governing the transplantability of tumors are applicable essentially to normal tissues, and Rous[2] has shown that the methods of inducing resistance to tumor transplantation apply also to normal tissue grafts. In his well known zig-zag experiments, Ehrlich[3] showed that the limit of growth of a tumor graft in a foreign species (rat tumors in mice, or *vice versa*) is from 7 to 9 days, but in no instance has such a graft been successfully implanted into a second individual of the foreign species without being first returned to the original or homologous species. One of us demonstrated that this restriction of growth does not apply to the embryo, since rat tissues are capable of growing over long periods and through successive transplantation in chicken embryos.[4]

The explanation of the strict specificity of tissue transplantability has been sought in various ways and led to not a few hypothetical views. A certain number of indubitable facts have been discovered, but whether or not they are to be regarded as the essential or primary

[1] Loeb, Leo, *Proc. Am. Phil. Soc.*, 1908, xlvii, 3; *J. Cancer Research*, 1917, ii, 135; *J. Med. Research*, 1920–21, xlii, 137.

[2] Rous, P., *J. Exp. Med.*, 1910, xii, 344.

[3] Ehrlich, P., *Arb. k. Inst. exp. Therap. Frankf.*, 1906, No. 1, 77.

[4] Murphy, Jas. B., *J. Exp. Med.*, 1913, xvii, 482.

cause of the phenomenon or merely the secondary or concomitant and attendant results has not yet been determined.

Thus, it has been noted that a short time after the implantation of a heteroplastic graft, the latter becomes surrounded by a zone of cells in which the lymphoid variety of cell is dominant; the intensity of the cellular reaction is roughly proportionate to the degree of relationship existing between the two species concerned in the experiment.

On the other hand, it has been found that with the rat tumor graft in chicken embryos, referred to above, the cellular reaction is much delayed and does not become evident until the 19th day of incubation and coincides with the appearance of degenerative changes in the graft, which now suffers rapid and ultimately complete absorption.[5] It is significant that the cellular reaction about, and subsequent disintegration of the graft are hastened by the implantation into the embryo of a fragment of spleen from an adult chicken.[6]

These findings indicate unmistakably that the lymphoid cell reactions are associated with the resistance mechanism, and the importance of this association is emphasized by the fact that adult animals deprived of their lymphoid tissue by repeated exposures to suitable small doses of x-rays, like the embryo, fail to destroy foreign tissue grafts, which not only continue to grow actively, but may be transferred repeatedly to other individuals which have been prepared for their reception by the x-ray treatment.[7] One would expect that an animal with its resistance so reduced that even inoculated foreign tissue survives and grows actively would prove to be non-resistant to transplants from homologous spontaneous tumors, but this is not the case. Grafts from a spontaneous tumor undoubtedly survived for a longer period in x-rayed than in untreated animals, but we did not succeed in transplanting a tumor, under these conditions, which failed to grow in untreated or normal animals. One instance has been reported of transplantation of a rat tumor in x-rayed animals which failed in untreated rats,[8] but this method falls far short of what might be expected.

[5] Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 181.
[6] Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 513.
[7] Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459.
[8] Chambers, H., Scott, G., and Russ, S., *J. Path. and Bact.*, 1920, xxiii, 384.

The site of tissue transplanation has received but little attention, and as a rule the experimental studies have been made on grafts in the subcutaneous tissue or muscle. New points for consideration have been introduced by recent experiments reported by Shirai,[9] who has employed the brain as the locus of heteroplastic tumor transplants, in which situation, according to him, grafted tissue grows as readily in an alien as in an homologous host. The important bearing of this observation on the question of the resistance mechanism has led us to undertake an investigation of the subject.

Heterologous Tumor Grafts in the Brain.

Since Shirai has not described adequately the procedure he employed, we had first to work out a practical method.

Method.—After etherization of the animal, the head is shaved and a midline incision made, enabling the skin and fascia to be retracted. A small hole of sufficient size to admit a No. 18 gauge trocar is made in the skull. The trocar is provided with a shoulder of metal about 3 mm. from the point, in order to limit the depth of penetration. The point is beveled slightly so as not to cause unnecessary damage to the brain tissue. The material for inoculation is loaded into the trocar and pushed into the brain by means of a plunger, after which the trocar is withdrawn slowly so as not to remove the graft. Bleeding may be stopped by pulling the fascia over the opening or by applying a small piece of muscle.

Mouse Tumors in the Brains of Rats.—The cerebellum was chosen as the location for the first inoculations, but for reasons not altogether clear, this proved to be unsatisfactory. Occasionally excellent growths resulted, but in many animals the graft remnant was found imbedded in a mass of reaction tissue similar to that which appears about an heteroplastic graft in the subcutaneous tissue. Implantations into the posterior lateral part of the cerebrum also failed to give uniform results, because as was found, a graft lying in the ventricle or even coming into contact with the ventricle leads to a similar reaction. On the other hand, when the graft lay entirely in the brain substance, it grew almost invariably with great rapidity.

This observation led us to select the anterior part of the frontal lobe on account of the thickness of the cortex and the shallowness of the ventricle at this point. Grafting in this region gave 80 to 90 per cent

[9] Shirai, Y., *Japan Med. World*, 1921, i, 14, 15.

of tumors in the animals inoculated. In practically every instance in which the implant failed to grow, examination showed that it had come into contact with the ventricle.

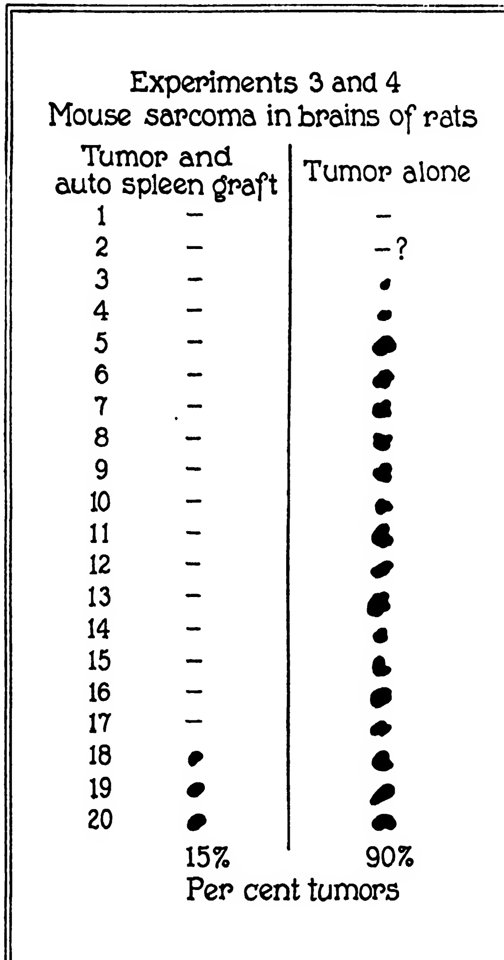
The growth resulting from inoculation of the rat brain with mouse sarcoma is generally a discrete, rounded nodule, pushing the brain substance away, rather than invading it directly. The tumors are copiously supplied with blood vessels and rarely show considerable areas of necrosis, which is in contrast with nodules developing in the subcutaneous tissue of the native host, in which the entire center of the growth is often necrotic. The number of mitotic figures is remarkable (Fig. 1). No cellular reaction is present about the edges of the typical growth lying entirely in the brain substance, but nearby vessels often show a pronounced collar of round cells (Fig. 2), and the lumina of small vessels are often blocked with lymphocytes. Other than exceptions to be mentioned, the cellular accumulations remain confined, and do not invade the brain tissue. If, in the course of growth, the tumor reaches the ventricle, the choroid plexus becomes enormously swollen and engorged with lymphocytes (Figs. 3 and 4). Under these circumstances the cells frequently invade the tumor, and necrosis of the portion of the tumor lying near the ventricle takes place (Figs. 5 and 6).

The mouse sarcoma used in these experiments grows with great rapidity in the brains of rats,—pin-head sized grafts producing tumors in 7 to 9 days which replaced the entire frontal lobe. This mouse tumor has also been grown in the brains of guinea pigs and pigeons. Moreover, a mouse carcinoma has been successfully implanted in the brains of rats, but the kind of growth obtained is somewhat different in that the tumors show a tendency to flatten on the surface of the brain and to extend apparently by invasion, sending finger-like processes into the brain (Fig. 7). In addition, necrosis is observed more frequently in the carcinoma than in the sarcoma, although less in extent than occurs in the subcutaneus tumor of the native host.

Effect of Autografts of the Spleen on the Growth of Foreign Tissue in the Brain.

As previously noted, an organism which is non-resistant to heteroplastic tissue, like the chick embryo, may be rendered resistant by a

graft of adult spleen. This, with the other evidence associating the lymphoid cell with the resistance mechanism, leads one to consider the possible importance of the observation that no cellular reaction



TEXT-FIG. 1.

occurs about a foreign tissue in the brain, and the following experiments were therefore undertaken to throw some light on this question.

Method.—The tip of the spleen of a rat was removed under ether anesthesia, and a small bit of the tissue, together with a graft of a mouse sarcoma, was im-

planted into the brain of the same rat. As a control, a graft of the tumor alone was introduced into the brain of another rat.

Of 50 rats receiving a graft of mouse sarcoma and an autograft of spleen, only eight developed tumors, and practically without exception these were small, often only nests of cells. On the other hand, 40 of the 48 controls developed tumors, and the growths generally replaced the entire frontal lobe of the brain (Table I and Text-fig. 1).

TABLE I.

Experiment No.	Tumor and auto spleen graft.		Tumor alone.	
	Takes.	No. of rats.	Takes.	No. of rats.
	per cent		*per cent*	
1	11.1	9	66.6	9
2	33.3	9	80.0	10
3	10.0	10	80.0	10
4	20.0	10	100.0	10
5	8.5	12	88.8	9
Average or total........	16.0	50	83.3	48

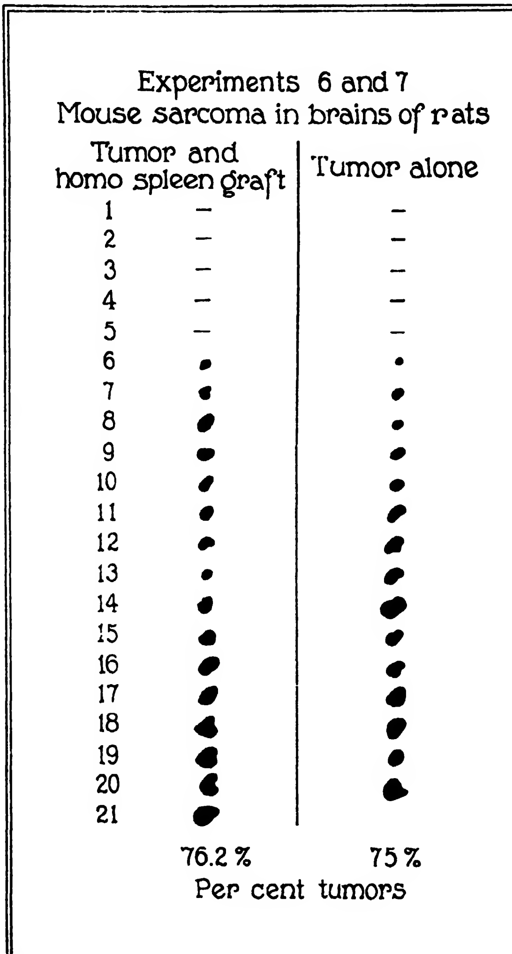
In two additional experiments, homografts of spleen instead of autografts, were introduced along with a graft of mouse sarcoma into the brains of rats, but absolutely no inhibitory action on the tumor was effected. Sixteen out of the twenty-one rats receiving tumor and homo spleen grafts developed sizable tumors, while in the controls with sarcoma alone, fifteen out of twenty had tumors (Table II and Text-fig. 2).

TABLE II.

Experiment No.	Tumor and homo spleen graft.		Tumor alone.	
	Takes.	No. of rats.	Takes.	No. of rats.
	per cent		*per cent*	
6	72.7	11	80.0	10
7	80.0	10	70.0	10
Average or total........	76.2	21	75.0	20

This complete lack of activity on the part of the homo spleen graft is in strong contrast to the almost complete inhibitory action of the

auto spleen grafts. As a further control it was shown that autoplastic testicular grafts exert no inhibitory action, nor does autologous blood injected with the tumor affect its development.[10] In the experiment with auto spleen graft reported above, it was noted that



TEXT-FIG. 2.

the lymphoid cells disappear from the fragment very soon after the death of the tumor cells, and it has also been noted that when an auto spleen graft is inoculated alone into the brain, the lymphoid elements

[10] Maisin, J., and Sturm, E., *Compt. rend. Soc. biol.*, 1923, lxxxviii, 1216.

disappear still more rapidly. In fact it is rare to find any surviving cells of this type after 48 hours, while the cells of a similar graft in the subcutaneous tissue survive for a longer time.

Homoplastic Tumor Grafts in the Brain.

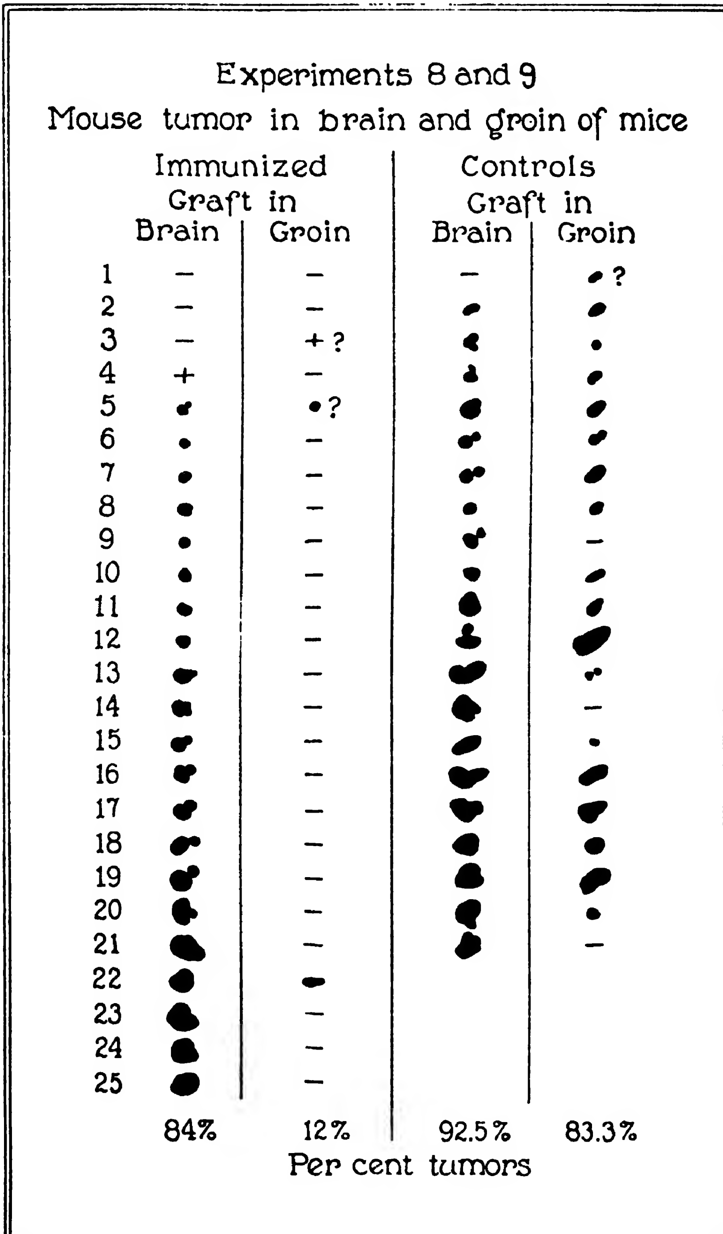
All the factors studied indicate that the mechanism involved in the destruction of the heteroplastic tissue graft is also responsible for the natural resistance possessed by a proportion of animals to homoplastic tumor grafts. Even induced resistance to tumor transplants shows itself locally by the same series of events as those which take place in natural resistance to both homo- and heteroplastic grafts. If this similarity is more than a superficial manifestation, we should expect, from the preceding observations, that animals highly resistant to homoplastic tumors, transplanted in the usual locations, would be susceptible to inoculation in the brain.

Experiment.—A number of mice were immunized against tumors by the injection of 0.2 cc. of defibrinated mouse blood, and 10 days later were inoculated with a transplantable mouse adenocarcinoma, both in the brain and in the subcutaneous tissue of the groin. As controls, normal mice were given similar inoculations with the same tumor. The results of six experiments carried out on 127 mice are given in Table III. Text-fig. 3 shows the results in two of the experiments.

TABLE III.

Experiment No.	Immunized mice.			Control mice.		
	Tumor in brain.	Tumor in groin.	No. of mice.	Tumor in brain.	Tumor in groin.	No. of mice.
	per cent	*per cent*		*per cent*	*per cent*	
8	80.0	10.0	10	100.0	77.7	9
9	86.6	13.3	15	91.6	83.3	12
10	91.6	25.0	12	72.7	81.8	11
11	88.8	12.0	9	100.0	90.0	10
12	100.0	22.0	9	100.0	80.0	10
13	90.0	30.0	10	90.0	80.0	10
Average or total........	89.2	21.5	65	91.9	82.2	62

It may be concluded from the results of these experiments that while the immunized mice show the usual high rate of immunity against subcutaneous grafts, the same animals have almost a complete


Experiments 8 and 9
Mouse tumor in brain and groin of mice
Immunized
Graft in
Brain
Groin
Controls
Graft in
Brain
Groin
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
84%
12%
92.5%
83.3%
Per cent tumors

TEXT-FIG. 3.

lack of resistance to similar grafts in the brain. Inoculations in the latter locality resulted in tumors in 89.2 per cent of the animals, which is practically the same rate as that shown in the brain of non-immunized controls and a higher rate than resulted from an inoculation in the groin of even the control mice.

The histological study of specimens from the preceding groups of animals brought out an interesting fact relating to the heteroplastic tissue experiment in that when a graft came in contact with the ventricle in a resistant mouse, a marked cellular reaction occurred, resulting in the complete or partial destruction of the tumor. On the other hand, grafts in susceptible mice frequently grew into the ventricle and invaded the choroid plexus without inducing a reaction greater than that seen about a subcutaneous graft in a susceptible animal.

In order to test the effect of auto and homo spleen grafts on the growth of tissue in the brains of immunized and non-immunized mice, a further investigation with homoplastic tissue was undertaken.

Experiment.—55 mice were immunized by the injection of 0.2 cc. of defibrinated mouse blood, and 10 days later, twenty-seven of these were inoculated in the brain with a mouse carcinoma and a bit of analogous spleen tissue. The remaining twenty-eight mice were inoculated in the brain with tumor alone. In addition, all the animals of the two groups were given a graft of tumor in the groin. As a control, normal mice were inoculated in the groin and in the brain with tumor alone.

In another experiment, normal mice were inoculated in the brain with autologous spleen grafts and tumors, and in a second series with homologous spleen and tumor. All animals received in addition a graft of the tumor alone in the groin, and control animals were also inoculated with the same tumor in the brain and in the groin. For comparison, the results of the two experiments are given in Table IV.

TABLE IV.

Experiments 14, 15, and 16.

Immunized mice.				Control mice.	
27 mice inoculated with		28 mice inoculated with		30 mice inoculated with	
Auto spleen graft and tumor in brain.	Tumor alone in groin.	Tumor alone in brain.	Tumor alone in groin.	Tumor alone in brain.	Tumor alone in groin.
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
29.6	25.9	89.3	31.4	96.6	83.3

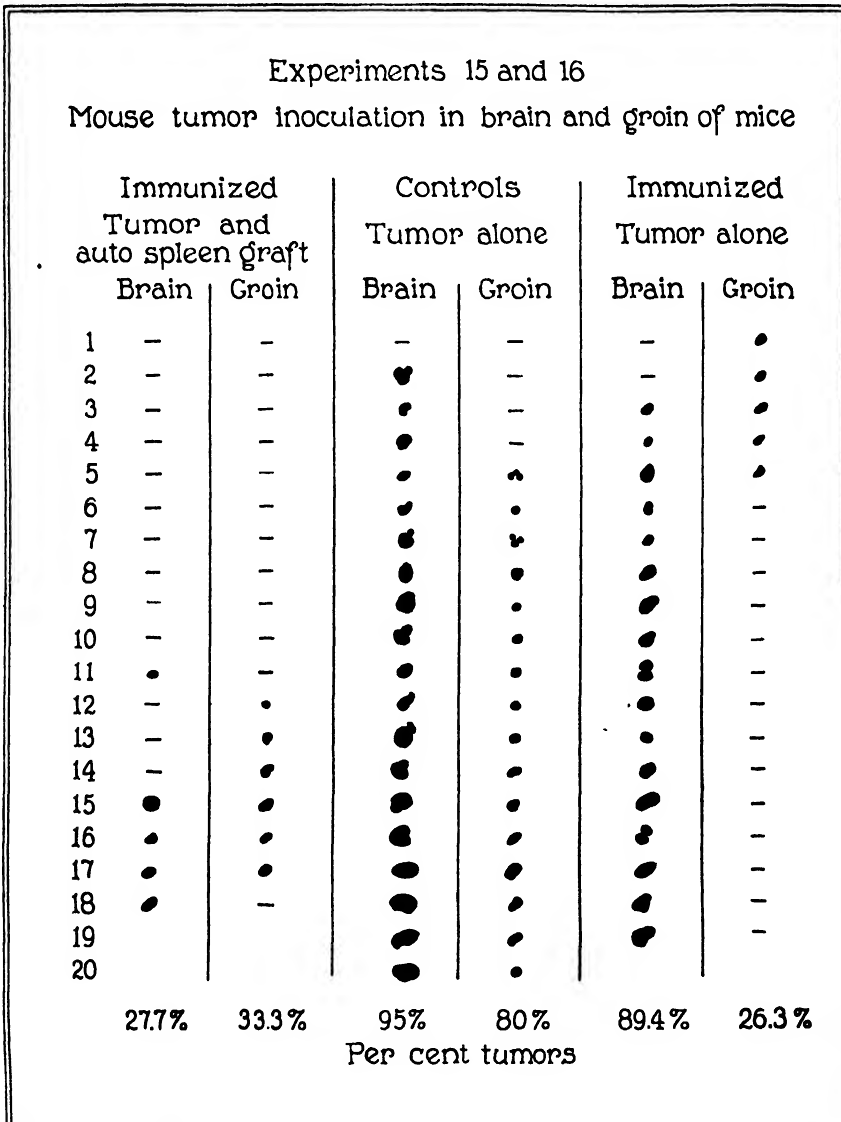
TABLE IV—*Concluded.*

Experiments 17 and 18.

Non-immunized mice.				Control mice.	
20 mice inoculated with		19 mice inoculated with		20 mice inoculated with	
Auto spleen graft and tumor in brain.	Tumor alone in groin.	Homo spleen graft and tumor in brain.	Tumor alone in groin.	Tumor alone in brain.	Tumor alone in groin.
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
55.0	75.0	84.2	84.2	95.0	70.0

It may be deduced from the above table that immunized mice inoculated into the brain with homologous spleen and tumor grafts have considerable resistance, while immunized mice inoculated with tumor alone are almost completely lacking in a defensive reaction to tumor growth (Text-fig. 4). Even non-immunized mice, inoculated with auto spleen and tumor grafts show some resistance, but much less than that exhibited by the immunized animals. In contrast to the effectiveness of the auto spleen graft in its inhibitory action is the almost complete inactivity of homo spleen grafts. In this respect we have another agreement in the behavior of hetero- and homoplastic grafts in the brain.[11]

[11] Some time ago we carried out an experiment to test the effect of auto- and homosplenic tissue on the fate of tumor grafts inoculated in the subcutaneous tissue, the splenic tissue in this experiment being mixed with the tumor cells before inoculation. The results based on the inoculation of 114 animals were 54.6 per cent only of takes with auto- and 80 per cent of takes with homoplastic spleen, while the controls gave 94 per cent of takes. This harmonizes with the above result.


Experiments 15 and 16
Mouse tumor inoculation in brain and groin of mice
Immunized
Tumor and auto spleen graft
Controls
Tumor alone
Immunized
Tumor alone
Brain
Groin
Brain
Groin
Brain
Groin
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
27.7%
33.3%
95%
80%
89.4%
26.3%
Per cent tumors

TEXT-FIG. 4.

Failure of Grafts of Spontaneous Tumors in the Brain.

With the usual methods the spontaneous tumors of animals have proved to be rarely transplantable even to animals of the same species, and in some species no successes have been recorded. As already noted, resistance to such transplants may be somewhat mitigated by exposing the animals to x-rays, a procedure which has made it possible to transplant at least one tumor which otherwise failed, but even this method was too capricious to be of real value. The seeming complete lack of resistance of the brain naturally led to a consideration of this locality as a site for the inoculation of spontaneous tumor, with the expectation that perhaps a larger proportion might be secured for study.

Some sixty rats have been inoculated into the brain with nine different spontaneous tumors of the mouse without a single success. Over a hundred mice have been similarly inoculated with seventeen different spontaneous mouse tumors and in only ten animals could any surviving tumor cells be found at the end of 10 days or 2 weeks. In only one or two instances was there anything like a tumor formation (Fig. 8), while in the others small islands only of tumor cells imbedded in reaction tissue remained.

DISCUSSION.

It is not clear why there should be an absence of a cellular reaction about a heteroplastic graft in the brain, but two possible explanations suggest themselves; first, that for mechanical reasons the cells are unable to migrate beyond the perivascular spaces; second, that the lymphoid cells find the brain tissue an uncongenial environment. In support of the first idea are the occurrence of extensive perivascular reactions near the implant, and also the observation that the small vessels in and near the tumors are frequently crowded with, and sometimes blocked by cells of the lymphoid type. In support of the second possibility is the fact that lymphoid cells implanted alone in the brain disappear more quickly than from the subcutaneous tissue. These points are suggestive only, for we have no real knowledge of what is responsible for the absence in the brain of the usual cellular reaction about a foreign tissue graft.

There seems little doubt that the absence of a reaction of this type is the principal reason for the failure of the brain to destroy heteroplastic tissues which are so readily taken care of in other locations in the body. This notion is strengthened by the observation that a foreign tissue graft in contact with the ventricle induces the typical cellular reactions and is as promptly combated as would be a similar graft in the subcutaneous tissue. Furthermore, the necessary resistance factor may be supplied to the otherwise passive brain by the inoculation of a bit of spleen tissue along with the foreign tissue graft, this being sufficient to suppress the growth of the latter,—a condition similar to that observed in the chick embryo. On the whole, it may be said that the results described confirm and extend our earlier views on the association of cellular reaction with the resistance mechanism in respect to heteroplastic grafting.

From our previous observations it was to be expected that in the absence of cellular reactions immunity to homoplastic tumors of the transplantable type would be lacking in the brain tissues, but the explanation for the failure of spontaneous cancer grafts to grow readily even in homologous animals is not clear and must await further experimentation. It is possible, of course, that this failure may be determined as much by some inherent quality of the cancer cells as by the resistance on the part of the new host.

SUMMARY.

In confirmation of Shirai's observation, we find that transplantable mouse tumors grow actively when inoculated into the brains of rats, guinea pigs, and pigeons, whereas subcutaneous or intramuscular grafts in the same animals fail. This growth of foreign tissue in the brain, however, takes place only when the grafted material lies entirely in the brain tissue; if it comes in contact with the ventricle a cellular reaction takes place with resultant destruction of the graft.

The growth of foreign tissue in the brain may be completely inhibited by simultaneous inoculations of a small bit of autologous but not by a bit of homologous spleen tissue.

Mice highly immune to subcutaneous transplants of mouse cancer show no resistance to such tumors when the inoculation is made into the brain.

Although the brain is without obvious power of resistance to implants of transplantable heteroplastic mouse tumors, yet grafts of spontaneous tumors fail to grow there even, as a rule, when tumor implanted and animal host are of the same species.

EXPLANATION OF PLATES.

Plate 13.

Fig. 1. A mouse sarcoma growing in the brain of a rat. *M*, mitotic figures.

Fig. 2. Perivascular infiltration in the brain of a rat, which occurs near a foreign tumor graft.

Plate 14.

Fig. 3. Choroid plexus engorgement with round cells resulting from the encroachment of a foreign tumor.

Fig. 4. The same as Fig. 3, but showing the normal plexus at a lower part of the ventricle.

Plate 15.

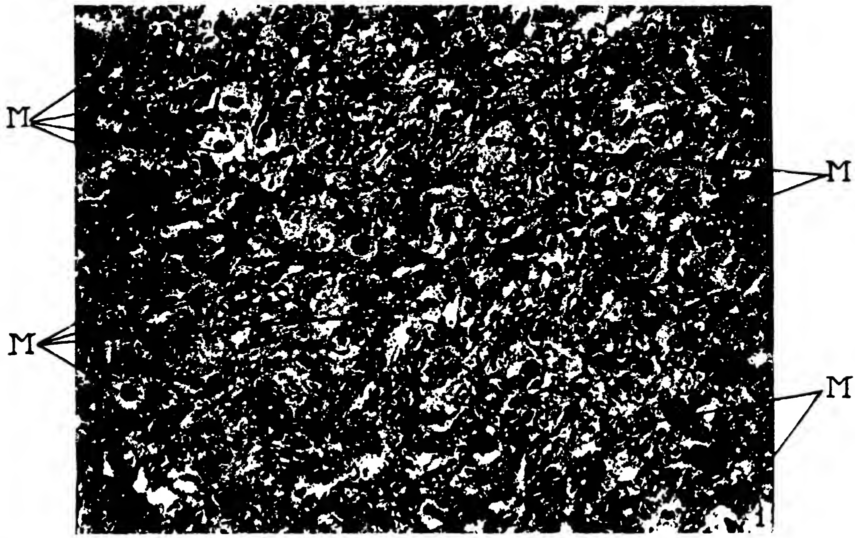
Fig. 5. Result of encroachment of a foreign tumor on the ventricle.

Fig. 6. The same as Fig. 5. The engorged choroid plexus is seen in the lower part of the section.

Plate 16.

Fig. 7. The growing edge of a mouse carcinoma in the brain of a rat.

Fig. 8. Spontaneous tumor of a mouse growing in the brain of another mouse.



(Murphy and Sturm: Transplantability of tissues in the brain.)

(Murphy and Sturm: Transplantability of tissues in the brain.)

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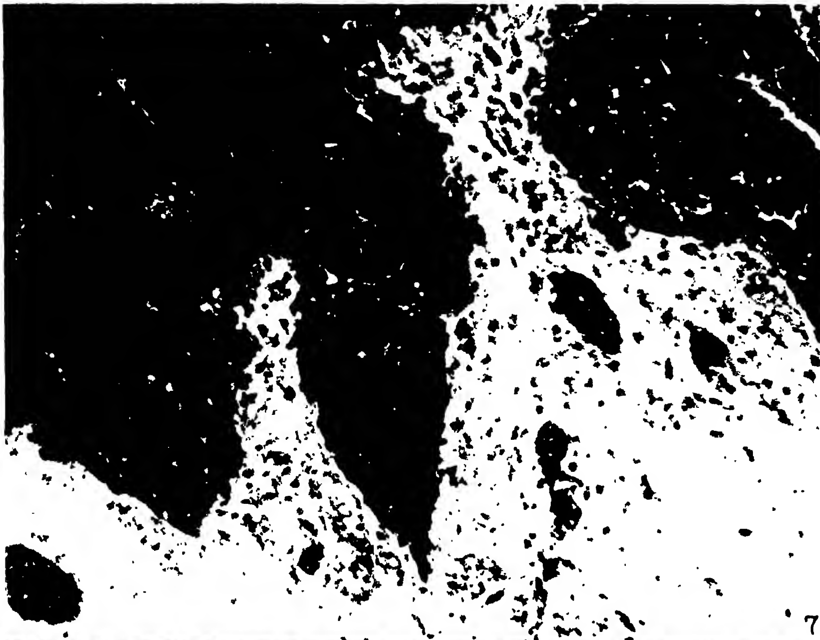
(Murphy and Sturm: Transplantability of tissues in the brain.)



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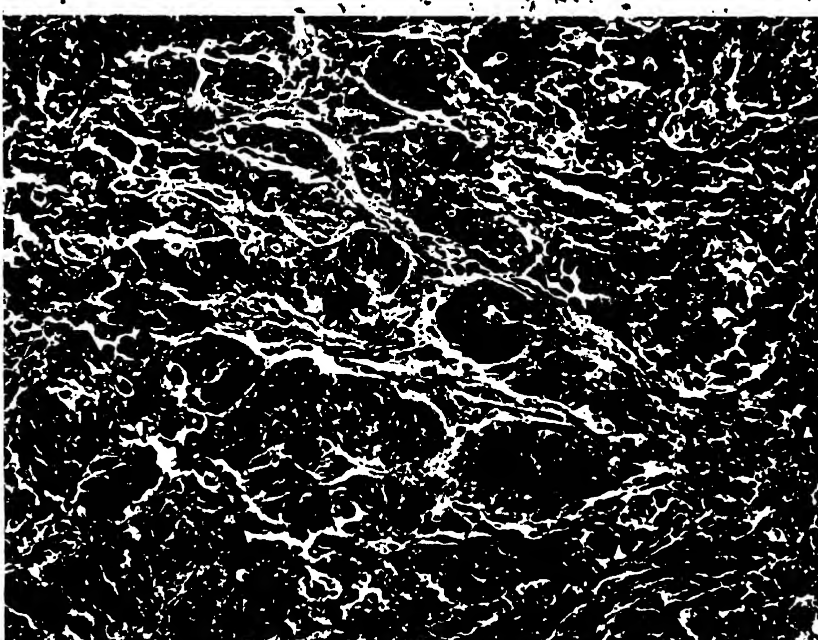

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(Murphy and Sturm: Transplantability of tissues in the brain.)

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STUDIES ON X-RAY EFFECTS.

XIII. HISTOLOGICAL STUDY OF THE FATE OF CANCER GRAFTS INOCULATED INTO AN X-RAYED AREA.

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PLATES 22 AND 23.

(Received for publication, May 28, 1923.)

The familiar phases of cancer cell degeneration following x-ray treatment in man have generally been attributed to the direct injury produced by the irradiation, but there is little experimental proof to support this idea. On the contrary, recent studies have indicated that the treatment dose of x-rays has little, if any, direct effect on the cancer cells, and that the curative action of this agent depends largely, if not entirely, on the reaction induced in the surrounding normal tissue.[1] The basis for this conclusion is to be found in the fact that a cancer graft will rarely grow when inoculated into a region which has previously been exposed to an erythema dose of x-rays, while grafts of the same tumor grow actively in unradiated parts of the same animal.

Under the conditions of an experiment such as that described above, the cancer cells inoculated into an x-rayed area themselves received no x-rays, and therefore any changes taking place in them must be secondary to the altered condition induced by x-rays in the surrounding tissue. The object of the present investigation is to examine histologically the series of degenerative changes taking place in cancer cells implanted in a location made unfavorable for their growth by a previous exposure to x-rays.

[1] Murphy, Jas. B., Hussey, R. G., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 299.

EXPERIMENTS.

Sixteen mice, after etherization, were shaved in the upper abdominal region down to and including both groins, and were then secured on a small board. An opening, measuring about 15 by 20 mm., was cut out of a piece of sheet lead, and this was placed over the animal so as to expose the left groin, to the midline, and completely protect the rest of the body. This area was then given a dose of x-rays governed by the following factors: 3 inch spark-gap, 10 milliamperes, 6 inch distance from the target, and time of exposure 2½ minutes.

7 days later, when a mild erythema was first appearing, cancer grafts (Bashford Adenocarcinoma No. 63) were inoculated intracutaneously in the x-rayed area and like grafts in the corresponding location of the right, non-radiated groin.

Six of the sixteen mice were killed, a pair at a time, 48 hours, 4 days, and 7 days after the cancer inoculation. The cancer grafts with the surrounding tissue in the x-rayed and protected groins were removed and fixed in Carnoy's chloroform-alcohol-acetic fluid. The remaining ten mice were killed in pairs 24 hours, 48 hours, 3 days, 5 days, and 7 days after cancer inoculation, and the grafts were fixed in formaldehyde. Sections were stained with methylene blue or hematoxylin and eosin.

In order to determine what would have been the outcome of the tumor inoculations, six additional mice were subjected to the same x-ray treatment and inoculations, but were allowed to live. In the course of 3 weeks five out of six inoculations in the protected area resulted in healthy tumors, while only one tumor developed out of six inoculations made in the x-rayed area.

Histological Description.

The grafts removed 24 hours after inoculation were found to consist of a necrotic mass with islands of healthy cancer cells scattered here and there. In and around the grafts there was an acute polymorphonuclear and a mild lymphoid reaction. In the condition of the inoculated material and the reaction immediately surrounding it, no difference could be discovered between the grafts from the

two sides, but in the x-rayed area there was a marked lymphoid reaction in the skin layers, which, however, did not come in direct contact with the graft.

In the x-rayed area the necrotic debris of the original mass of graft tissue had been largely removed at the end of the 48 hour and 3 day periods, and the islands of healthy looking cancer cells showed frequent mitotic figures and seemed larger than at the previous period. These islands often formed a more or less continuous ring around the remains of the necrotic mass. The polymorphonuclear reaction had almost subsided and was replaced by a more extensive reaction of the cells of the lymphoid variety (Fig. 1). There was also pronounced activity of the fibroblastic tissue, and the cellular infiltration of the skin layers was still very prominent. At the same period cancer grafts in the protected areas were found to be in a more active stage of growth, islands of cancer cells tending to coalesce and to form irregularly shaped masses of healthy looking tissue. The polymorphonuclear reaction was much reduced, as in the x-rayed area, while the lymphoid cell infiltration was now present to only a limited extent around the cancer grafts.

At the 4 to 5 day periods the difference between thecancer grafts in x-rayed areas and those in protected areas was that degenerative changes had become even more prominent in the former, while the latter continued to grow actively.

The first event in this degenerative process, as seen under the microscope, was the swelling of both nucleus and cytoplasm. The cytoplasm gradually became more acidophilic, and the nucleus hyperchromatic (Fig. 2). The nucleus finally lost its structure, became more deeply stained, and later uniformly pycnotic. The general cell structure also became less and less distinct, and with the fragmentation of the pycnotic nuclei the cells were reduced to debris.

It was observed also that two or more cells often coalesced to form giant cells with highly vacuolated cytoplasm (Figs. 3 and 4), this type of change being found more frequently in small groups which had become imbedded in a fibrous matrix (Figs. 3, *A* and 4, *B*). It was not uncommon to find the nuclei pushed to the periphery of some cells by large masses of inclusion bodies (Fig. 3, *B*). In later stages of degeneration the nuclei lost their staining capacity and

appeared as the so called "ghost nuclei" (Fig. 4, *B*), and then finally became unrecognizable, leaving the cells as irregularly shaped masses of homogeneous appearance.

This degeneration process was generally complete by the 7th day, when the remains of the graft in the x-rayed area were represented by a small mass of necrotic debris, attended by some polymorphonuclear cells and macrophage reaction.

DISCUSSION.

It may be noted that the series of changes described above are in every respect similar to those that have been reported by Apolant,[2] Contamin,[3] Clunet,[4] Marie and Clunet,[5] Colwell and Russ,[6] Knox,[7] and others, as the typical changes taking place in cancer cells following radiation. To summarize the statements of these authors, all of whom describe essentially the same series of events, the first change occurring after the irradiation of cancer is a lymphocytic and to a less extent a polymorphonuclear exudation, and a connective tissue proliferation around the neoplasm. A few days later the cancer cells themselves begin to show signs of degeneration. These degenerative changes entail a marked swelling of both nucleus and cytoplasm, loss of structural details, hyperchromatism, pycnosis, and finally fragmentation of the nucleus, appearance of vacuoles, with increase in acidophilic affinity of cytoplasm, and giant cell formation by cell fusion. As the lymphocytes and connective tissue stroma increase, the cancer tissue is broken up into small islands, and at the final stage the pycnotic nuclei disintegrate and all traces of living cells are lost.

[2] Apolant, H., *Deutsch. med. Woch.*, 1904, xxx, 454.

[3] Contamin, M. A., *Compt. rend. Acad.*, 1910, cl, 1537.

[4] Clunet, J., cited by Woglom, W. H., The study of experimental cancer, a review, New York, 1913.

[5] Marie, P. L., and Clunet, J., cited by Woglom, W. H., The study of experimental cancer, a review, New York, 1913.

[6] Colwell, H. A., and Russ, S., Radium, x-rays and living cells, London, 1915.

[7] Knox, R., Radiography, x-ray therapeutics, and radium therapy, New York, 1916.

The general deduction has been that these characteristic changes resulted from the direct injury inflicted on the cancer cells by the x-rays. That this deduction is open to question is shown by the facts presented here; namely, that cancer cells implanted in an area of skin previously exposed to x-rays undergo exactly the same series of degenerative changes as those supposed to result from the direct injury.

In the case of radium treatment, especially when the emanation tube is imbedded in cancer tissue, there is little doubt that some of the cancer cells are directly killed by this agent. Recent observations by Ewing[8] and Alter[9] suggest, however, that the direct injury alone can hardly account for the entire process, and, furthermore, the effect of imbedded emanation tube on normal tissue as reported by Bagg[10] indicates that the local accumulation of lymphocytes, rather than the necrosis of tissues immediately surrounding the tube, may be the more extensive of the induced changes. It would seem probable, therefore, that with radium as well as with x-rays, the induced reaction in the surrounding tissue plays the important rôle in bringing about the destruction of the cancer cells.

SUMMARY.

Cancer cells implanted in a skin region previously exposed to an erythema dose of x-rays show a series of degenerative changes in every way comparable to the frequently described stages of cancer cell degeneration following x-ray treatment. The findings contrast strongly with the survival and growth of grafts implanted in unexposed regions in the same animal. Since the changes are the same whether the cancer cells have been directly exposed *in situ* or merely implanted in the previously exposed skin, it follows that it is impossible to establish microscopically a direct injury from the x-raying as the principal factor in the therapeutic action of x-rays on cancer.

[8] Ewing, J., *J. Am. Med. Assn.*, 1917, lxviii, 1238.
[9] Alter, N. M., *J. Med. Research*, 1919, xl, 241.
[10] Bagg, H. J., *Am. J. Roentgenol.*, 1920, vii, 536.

EXPLANATION OF PLATES.

Plate 22.

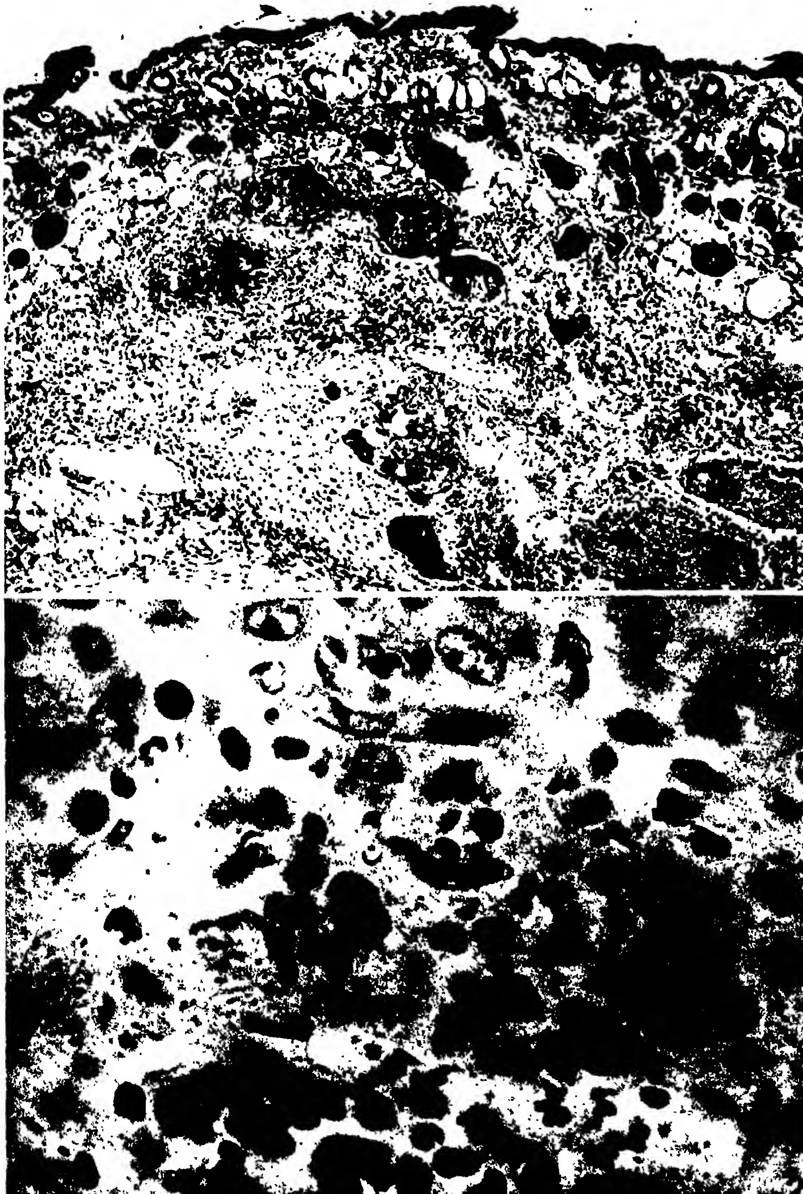
Fig. 1. 48 hour cancer graft in the area of skin exposed to an erythema dose of x-rays 7 days previous to cancer inoculation. Small islands of cancer cells are shown in the midst of the skin layers thickly infiltrated, principally by cells of the lymphoid variety.

Fig. 2. Part of a 5 day graft in x-rayed skin. Hyperchromatism, pycnosis, and fragmentation of nuclei are represented. Some healthy living cancer cells are shown at the top.

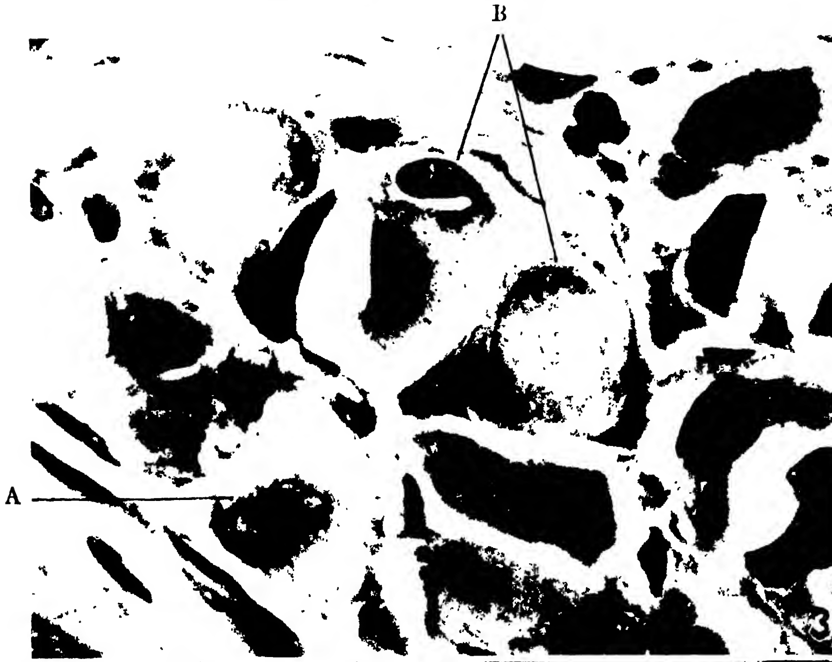
Plate 23.

Fig. 3. A small group of cancer cells imbedded in fibrous tissue, showing binucleated and highly vacuolated giant cell (*A*) and giant cells with nuclei pushed to the periphery (*B*).

Fig. 4. The same as Fig. 3, showing a binucleated giant cell (*A*) and so called "ghost cells" (*B*).



(Nakahara: Studies on x ray effects. XIII.)



(Nakahara: Studies on x-ray effects. XIII.)

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STUDIES ON LYMPHOID ACTIVITY.

VII. SUPPRESSION OF INDUCED IMMUNITY TO TRANSPLANTED CANCER BY LARGE DOSES OF OLIVE OIL.

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(Received for publication, June 7, 1923.)

As previously reported, a small quantity of olive oil injected intraperitoneally in mice induces a typical form of immunity to transplantable cancer.[1] Incidentally, it was noted that olive oil, while in small doses increases resistance, in sufficiently large doses renders mice hypersusceptible to the growth of cancer transplants. This quantitative relation presents an interesting parallel to the action of x-rays, with which agent, by changing the dosage properly,[2–4] the resistance of mice to transplantable cancer can almost at will be either increased or decreased.

An artificial immunity can also be broken down by means of comparatively massive doses of x-rays.[3,5,6] The present study was undertaken to determine whether or not olive oil also is capable of setting aside the state of so called induced immunity to transplanted cancer.

Effect of Olive Oil Injected into Immunized Mice Immediately before Cancer Inoculation.

In all the experiments to be reported here, commercial olive oil, described as the first expression, was used. Injections were made

[1] Nakahara, W., *J. Exp. Med.*, 1922, xxxv, 493.

[2] Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459.

[3] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

[4] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800. Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 429; 1922, xxxv, 475.

[5] Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1.

[6] Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917–19, xc, 1.

intraperitoneally in amounts of 0.7 cc. each, which dose was found adequate, although a larger quantity, even up to 1 cc., can be safely administered in a single injection. The mice for the experiments were young adults from one stock, and all of about the same size. Bashford Adenocarcinoma No. 63 was employed for transplantations.

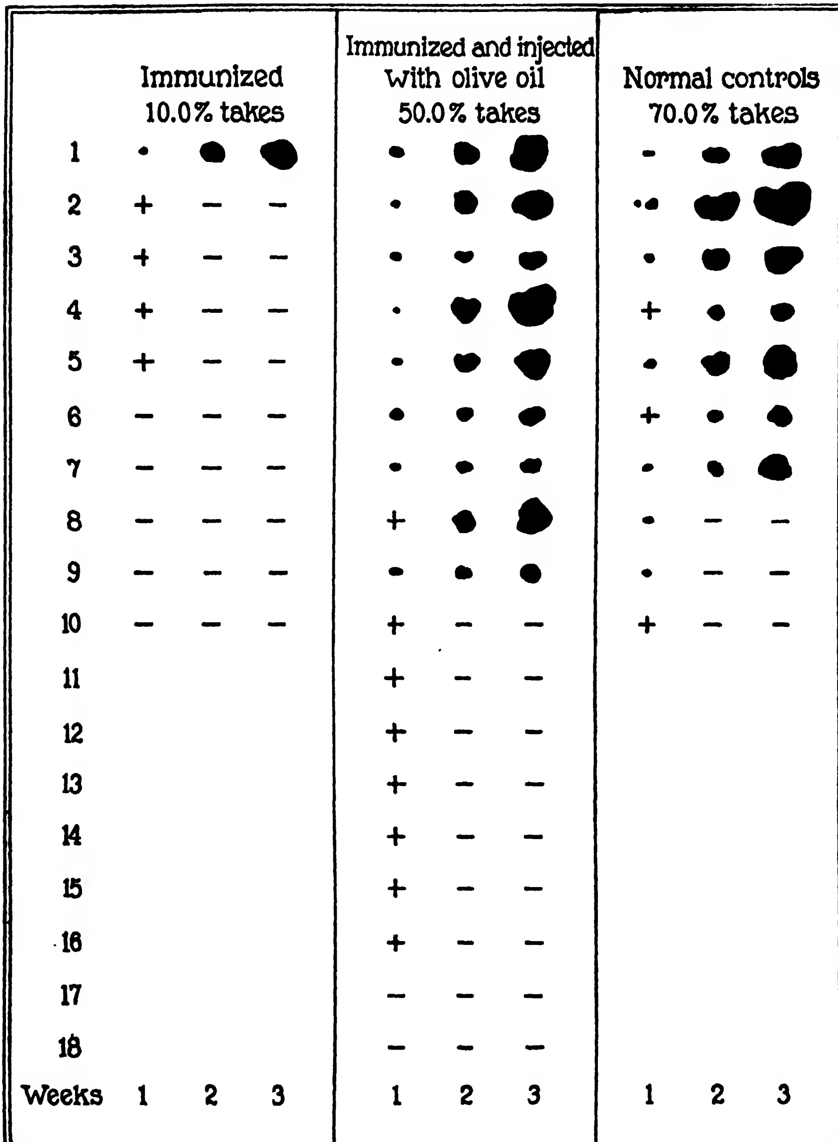
Experiments 1 to 4.—An immunizing injection of 0.2 cc. of defibrinated mouse blood was given to a number of mice which were divided into two groups. 10 days later those of one group were injected with olive oil, and immediately afterward inoculated with bits of the Bashford tumor. The other group, and a suitable number of normal, untreated mice, were inoculated with the tumor at the same time (Table I).

TABLE I.

Experiments 1 to 4.

Experiment No.	Takes.		
	Group 1. Immunized.	Group 2. Immunized and oil-injected.	Group 3. Controls.
1	22.2 per cent (9 mice).	71.5 per cent (7 mice).	100.0 per cent (9 mice).
2 (Text-fig. 1).	10.0 per cent (10 mice).	50.0 per cent (18 mice).	70.0 per cent (10 mice).
3	0.0 per cent (9 mice).	35.3 per cent (17 mice).	44.4 per cent (9 mice).
4	25.0 per cent (8 mice).	65.0 per cent (20 mice).	
Average.............	13.9 per cent (36 mice).	53.2 per cent (62 mice).	71.4 per cent (28 mice).

The results show conclusively that by injecting a large quantity of olive oil in potentially immune mice, immediately before cancer inoculation, the number of takes in these mice can be so increased as to approach that in normal controls. The percentage of takes was far greater than in mice immunized but given no olive oil.



TEXT-FIG. 1. Relative sizes of tumors in Experiment 2. Cancer inoculation was made in the immunized group 10 days after immunization; in the immunized and oil-injected group 10 days after immunization and immediately after olive oil injection.

Duration of the Suppressive Effect of Olive Oil on Potential Immunity.

The next experiments dealt with the duration of the suppressive effect of the olive oil injection.

Experiments 5 to 7.—Mice were immunized and divided into two groups as in preceding experiments. 10 days later one group was injected with olive oil, while the other group received no injection. 17 days after the immunizing blood injection, and 7 days after olive oil injection, all the mice were given tumor grafts, as were a number of normal mice. The results are shown in Table II.

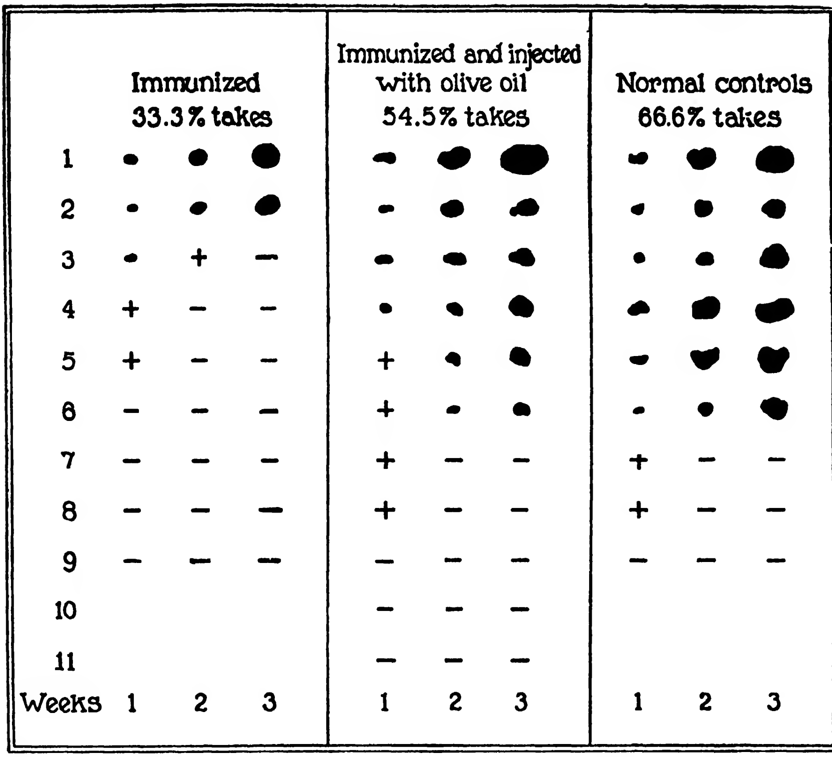
TABLE II.

Experiments 5 to 7.

Experiment No.	Takes.		
	Group 1. Immunized.	Group 2. Immunized and oil-injected.	Group 3. Controls.
5 (Text-fig. 2).	33.3 per cent (9 mice).	54.5 per cent (11 mice).	66.6 per cent (9 mice).
6	40.0 per cent (10 mice).	70.0 per cent (10 mice).	75.0 per cent (8 mice).
7	30.0 per cent (10 mice).	55.5 per cent (9 mice).	70.0 per cent (10 mice).
Average.............	34.5 per cent (29 mice).	60.0 per cent (30 mice).	70.3 per cent (27 mice).

It is evident from the above figures that the depressive effect of olive oil on the resistance is still operative 7 days after the injection. Although there is some falling off in the general effect of the induced resistance, there is still considerable difference between the number of takes in the immunized group which was given oil and the one which was not. As the duration of the induced immune state is limited to a few weeks,[7] it was considered unprofitable to make a test at a later period.

[7] Bashford, E. F., *Brit. Med. J.*, 1906, ii, 209. Bashford, E. F., Murray, J. A., and Cramer, W., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 164. Woglom, W. H., *J. Exp. Med.*, 1912, xvi, 629.



TEXT-FIG. 2. Relative sizes of tumors in Experiment 5. Cancer inoculation was made in the immunized group 17 days after immunization; in the immunized and oil-injected group 17 days after immunization and 7 days after oil injection.

Effect of Olive Oil Injected into Immunized Mice after Cancer Inoculation.

Further experiments were undertaken to determine whether the immunity process, already called into action by a cancer inoculation in potentially immune mice, can be influenced by olive oil injection. As the destruction of cancer grafts in immune hosts is often complete in a short time, the oil injections in these experiments were made 24 hours after the cancer grafting.

Experiments 8 and 9.—Mice were immunized by the injection of blood as before, and 10 days later were inoculated with a tumor, together with a suitable number of normal controls. 24 hours after the cancer inoculation half the immunized mice were inoculated with olive oil. Table III shows the effects.

TABLE III.

Experiments 8 and 9.

Experiment No.	Takes.		
	Group 1. Immunized.	Group 2. Immunized and oil-injected.	Group 3. Controls.
8	10.0 per cent (10 mice).	10.0 per cent (10 mice).	70.0 per cent (10 mice).
9	30.0 per cent (10 mice).	20.0 per cent (10 mice).	70.0 per cent (10 mice).
Average..............	20.0 per cent (20 mice).	15.0 per cent (20 mice).	70.0 per cent (20 mice).

The results are of interest in showing that although it may be possible to increase the per cent of takes in immunized mice by administering larger doses of olive oil, the amount sufficient to affect the potential immunity is insufficient to exert any perceptible influence on the active immunity; that is, while it is possible to prevent the development of the resistance mechanism, it is impossible, apparently, at least by these means, to suppress it after it has been activated by cancer inoculation.

Modification of Cellular Reactions Accompanying Immunity.

Characteristic cellular manifestations of three varieties are now known to accompany the resistant state of animals to transplanted cancer: (1) marked lymphocytic infiltration around the inoculated grafts;[1,8] (2) stimulation of cell division in germ centers of lymphoid organs;[9] and (3) hyperlymphocytosis in the blood.[3] It is of interest

[8] Burgess, A. M., *J. Med. Research*, 1909, xxi, 575. Da Fano, C., *Z. Immunitätsforsch., Orig.*, 1910, v, 1. Rous, P., and Murphy, Jas. B., *J. Exp. Med.*, 1912, xv, 270. Tyzzer, E. E., *J. Med. Research*, 1915, xxxii, 201.

[9] Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.

to ascertain the effect of olive oil injections on these associated cellular reactions in instances in which the dose used was sufficient to suppress the immunity itself.

Experiment 10.—Ten normal white mice were immunized by subcutaneous injection of 0.2 cc. of mouse blood. 10 days later, they were injected intraperitoneally with 0.7 cc. of olive oil and immediately thereafter inoculated with fragments of the Bashford adenocarcinoma. These mice were killed in pairs, at intervals of 24 hours, 48 hours, 3 days, 4 days, and 5 days after the inoculation.

For comparison, ten normal mice were immunized as above, and 10 days later grafts of the tumor were similarly inoculated, but without the preceding injection of olive oil. These mice were killed, two at a time on 5 successive days as in the preceding instance.

The cancer grafts and surrounding tissue, the spleen, and lymph nodes were removed at autopsy, preserved in Carnoy's fluid, and prepared for microscopic study.

Local Lymphoid Infiltration.—The absorption of the exudate resulting from the trauma of inoculation was generally complete within 48 hours, after which only slight evidence of cellular reaction was seen in the tissue surrounding the cancer graft in the oil-injected series. During the early stages there were but few lymphocytes immediately surrounding the cancer graft, and a very slight degree of connective tissue proliferation. Occasionally, however, small groups of lymphocytes were present around blood vessels. Later, the connective tissue stroma became more abundant, the graft received a copious vascular supply, and lymphocytic infiltration also appeared, but only to a slight extent.

Cancer grafts from the series which were immunized and cancer-inoculated, but not given an olive oil injection, offered a marked contrast to the picture described above. There was a dense cellular infiltration of the tissue surrounding the cancer graft which was composed of lymphocytes, plasma cells, and fibroblasts, and in addition a perivascular reaction occurred about small vessels in the loose connective tissue beyond the edge of the main reaction, a typical manifestation of local reaction in cancer immunity.

Lymphoid Organs.—Histological study of the spleen and lymph nodes from the two series of animals showed no definite differences. The general impression derived therefrom was that in the animals

which had received oil, proliferation of the lymphoblasts in the germ centers was less intense than in the animals which had received no oil, but this difference, if present, was slight.

Blood Lymphocytosis.—The following experiments were carried out to determine the effect of the olive oil injection on the blood picture which ordinarily accompanies the immunity process.

Experiment 11 (Text-Fig. 3). Series A.—Counts were made on eight mice before and at intervals after immunization and cancer implantation, repeating, for the sake of comparison, the original observation of Murphy and Morton.[3] Of these eight mice, all but two proved to be immune to cancer inoculation (Table IV).

TABLE IV.

Lymphocyte Counts on Series A (Immunized).

Mouse No.	1 day before blood injection.	3 days after cancer inoculation.	10 days after cancer inoculation.	Outcome of cancer inoculation.
1	9,534	10,445	7,613	+
2	12,364	11,181	9,858	+
Average for susceptible mice....	10,949	10,813	8,735	
3	9,106	16,862	14,703	–
4	6,528	19,759	12,483	–
5	8,827	9,445	14,468	–
6	11,496	16,855	12,695	–
7	10,024	13,344	10,719	–
8	6,287	12,854	15,411	–
Average for immune mice.......	8,711	14,853	13,413	
Average for all the mice........	9,273	13,843	12,368	75.0 per cent immune.

Series B.—Counts were made on twenty mice immunized and grafted with cancer as in Series A, and in addition injected with 0.7 cc. of olive oil immediately preceding the cancer inoculation. In this series, thirteen out of twenty mice developed tumors (Table V).

Absolute counts of the lymphocytes, including large and small lymphocytes, large mononuclear cells, and transitionals, per cubic millimeter of blood in the above series of mice, are given individually in Tables IV and V, and the averages are charted in Text-fig. 3. Polymorphonuclear counts have not been included because they showed no material variation.

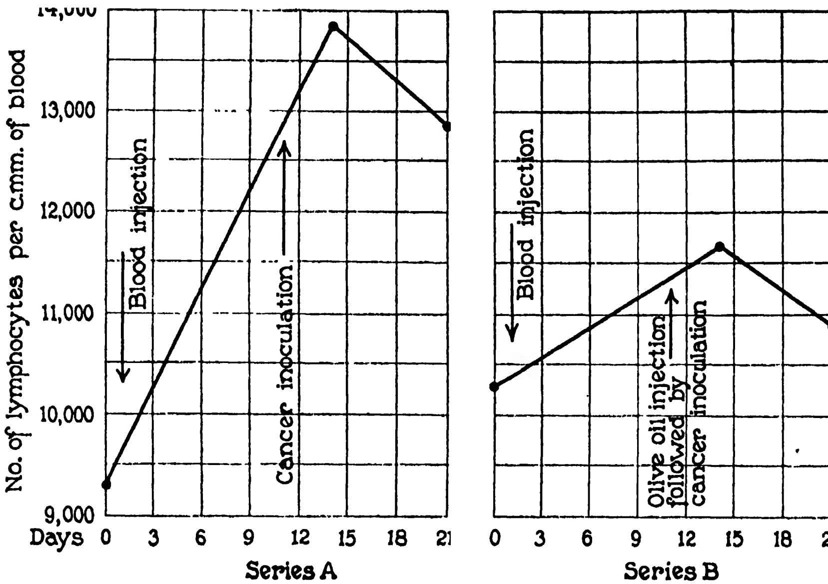
The lymphocytic crisis in the blood which usually develops in mmunized mice following cancer inoculation is seriously interfered with by olive oil injection. This fact is evident from a comparison

TABLE V.

Lymphocyte Counts on Series B (Immunized and Oil-Injected).

Mouse No.	1 day before blood injection.	3 days after cancer inoculation.	10 days after cancer inoculation.	Outcome of cancer inoculation.
1	10,473	8,894	8,675	+
2	6,578	7,249	6,555	+
3	10,381	10,903	10,087	+
4	12,276	8,711	8,648	+
5	7,741	7,613	7,076	+
6	10,332	10,451	8,627	+
7	9,778	11,198	9,936	+
8	11,838	13,239	12,282	+
9	10,331	6,986	9,104	+
10	6,980	9,615	9,679	+
11	8,659	6,673	6,128	+
12	13,848	10,539	11,833	+
13	17,042	17,153	10,685	+
Average for susceptible mice....	10,481	9,940	9,178	
14	12,221	21,386	18,195	–
15	13,868	15,733	18,316	–
16	11,026	15,377	16,243	–
17	5,660	13,210	12,506	–
18	7,694	14,358	11,334	–
19	13,442	16,896	12,921	–
20	5,752	8,890	8,812	–
Average for immune mice.......	9,951	15,121	14,046	
Average for all the mice........	10,296	11,753	10,882	35.0 per cent immune.

of the average variation of lymphocytes of Series A and B (Text-fig. 3). In Series B a small proportion of mice showed a lymphocytosis in spite of olive oil injection, and these mice later proved to be immune to the inoculated cancer.



TEXT-FIG. 3. Experiment 11. Composite curve of the lymphocyte counts on eight mice immunized and inoculated with cancer 10 days later (Series A), and on twenty mice immunized and 10 days later injected with olive oil followed by cancer inoculation (Series B).

Histological Changes in Normal Mice after Injection of a Large Dose of Olive Oil.

With thesuppressive action of olive oil on induced cancer immunity and on certain of its associated cellular reactions established, it has become desirable to ascertain the effect of such injections on normal mice. In an earlier communication,[1] a preliminary observation was noted; namely, that intraperitoneal injections of olive oil in large doses suppress the proliferation of lymphoid cells. The object of the next experiment has been primarily to repeat the work with a view to substantiating this point.

Experiment 12.—Ten normal mice were injected with 0.7 cc. of olive oil intraperitoneally, and were killed in pairs 48 hours, and 4, 7, 10, and 14 days after the injection.

Peritoneal Cavity.—It was noted, at autopsy, that the peritoneal cavities of all the mice were filled with unemulsified oil, in which only a very few cells could be seen microscopically.

Spleen.—At the 48 hour period the germ centers were found to be very small and showed an almost complete suppression of mitosis. The Malpighian bodies themselves were also much reduced in size, but there was no unusual amount of necrosis. Such changes could not be detected at the later periods.

Lymph Nodes.—The changes here were much the same, apparently, as those in the spleen; no unusual condition other than a transitory suppression of mitosis in the germ centers was detected.

Suprarenal Glands.—In every mouse killed 48 hours, and 4 days after the injection, some mitotic figures were seen among the cells of the cortex. On the average, one dividing cell was found for every two sections, but occasionally two were seen in the same section. At later periods no mitotic figures were found, and the condition appeared normal.

Other Organs.—Liver, kidney, bone marrow, and thymus and thyroid glands were examined, but showed no noticeable change.

The above findings corroborate the results previously reported as concerns the lymphoid changes, and they add an interesting manifestation in the suprarenal gland. The significance of the latter change is not apparent at present.

DISCUSSION.

The effect of large doses of olive oil on the lymphoid tissue differs from the x-ray effect, in that the former brings about merely a suppression of activity of the cells, while the latter actually destroys them. Yet olive oil is very nearly as potent in its interference with immunity to engrafted cancer as are x-rays.

It has often been suggested that induced resistance to cancer is a phenomenon closely related in type to the anaphylactic reaction. This notion is somewhat strengthened by the fact that x-rays not only destroy resistance to transplanted cancer,[3] but also prevent, as shown by von Heinrich[10] and by Hussey,[11] the sensitization of an

[10] von Heinrich, H., *Centr. Bakt., 1te Abt., Orig.*, 1913, lxx, 421.

[11] Hussey, R. G., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 22.

animal to a foreign protein. Unsaturated fatty acids are likewise capable of interfering with the development of sensitiveness to foreign proteins.[12] The present experiments demonstrate that olive oil, rich in unsaturated fatty acids, is also capable of annulling the immunity reaction to transplanted cancer.

SUMMARY.

The state of potential immunity to transplanted cancer engendered in mice by an injection of homologous blood may be reversed and converted into a susceptible state by an intraperitoneal injection of olive oil prior to the cancer inoculation.

The suppression of resistance is accompanied by the failure of certain cellular reactions known to develop during the establishment of immunity to transplanted cancer.

[12] Jobling, J. W., and Petersen, W., *J. Exp. Med.*, 1914, xx, 468.

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ACTION OF SERUM ON FIBROBLASTS IN VITRO.

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(Received for publication, January 17, 1923.)

I.

INTRODUCTION.

It had been observed previously that fragments of connective tissue, cultivated in plasma, did not increase in mass in spite of the activity they displayed in their medium. The growth of fibroblasts was no more extensive in serum than in media containing a small amount of serum or no serum at all, even when embryonic tissue juice was added to the medium.[1] Under the conditions of the experiments, serum never increased cell activity. As the action of serum had been determined merely by the rate of cell migration during a short time,[1] it was necessary to ascertain whether the duration of life of pure cultures of fibroblasts was modified by the presence of serum in varied dilutions and by its omission.

II.

EXPERIMENTAL.

The tissues were obtained from a 10 year old strain of fibroblasts,[2] and the serum from the plasma of young adult chickens which had fasted for 48 hours. The fibroblasts were cultivated in a medium of fibrinogen suspension,[3] Tyrode solution, and in varied amounts of serum. The medium was modified in different ways by substituting for Tyrode solution the requisite amounts of serum or tissue juice, according to the nature of the experiments. In order to prevent

[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.

[2] Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755.

[3] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.

the digestion of the fibrin in a serum-free medium, a small amount of egg yolk was added. Coagulation of the fibrinogen suspension was brought about by embryonic tissue juice in a concentration of 1:200. The composition of the medium was as follows:

Fibrinogen suspension	1 volume.
Doubly concentrated Tyrode solution containing 1 per cent egg yolk	1 volume.
Tyrode solution containing 1:50 embryonic tissue juice	1 volume.
Tyrode solution	1 volume.

TABLE I.

Influence of 0 Per Cent and 7 Per Cent Serum on the Duration of Life of Homologous Fibroblasts.

Passage No.	Culture No.	Experiment 1. Relative increase.			Experiment 2. Relative increase.			Experiment 3. Relative increase.			Experiment 4. Relative increase.		
		Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.
1	340	3.38	3.28	0.97	3.53	3.38	0.96	3.93	4.06	1.03	3.67	3.46	0.94
2	351	2.12	2.62	1.23	2.74	2.33	0.85	2.33	2.37	1.02	2.27	2.23	0.98
3	366	1.59	1.60	1.00	2.13	1.78	0.84	2.23	2.04	0.92	1.90	1.83	0.96
4	376	1.40	1.50	1.07	0	1.85		0	1.80	0	0	1.34	
5	386	1.00	1.12	1.12		0.85			0			0	
6	398	0	0.47	0		0							

Passage No.	Culture No.	Experiment 5. Relative increase.			Experiment 6. Relative increase.			Experiment 7. Relative increase.			Experiment 8. Relative increase.		
		Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.
1	325	3.62	3.80	1.05	4.21	4.21	1.00	3.25	3.69	1.13	3.60	3.00	0.83
2	339	3.48	3.55	1.02	3.29	3.50	1.06	3.03	3.13	1.03	2.69	2.82	1.05
3	350	2.13	2.21	1.04	2.00	2.00	1.00	1.54	1.58	1.03	1.69	1.48	0.87
4	365	1.89	1.66	0.88	1.81	1.72	0.95	1.73	1.62	0.94	1.78	1.62	0.91
5	375	1.70	1.50	0.88	1.60	1.00	0.63	0	0.53	0	2.00	1.85	0.93
6	385	0	0	0	1.50	0	0				1.50	1.30	0.87
7											0	0	0

The medium was modified by adding serum or tissue juice to replace a similar amount of Tyrode solution. The pH of Tyrode solution was 8.0, that of serum and tissue juice from 7.0 to 7.6, and that of fibrinogen suspension 6.8.

TABLE II.

Influence of 0 Per Cent and 25 Per Cent Serum on the Duration of Life of Homologous Fibroblasts.

		Experiment 1.			Experiment 2.			Experiment 3.			Experiment 4.		
		Relative increase.			Relative increase.			Relative increase.			Relative increase.		
Passage No.	Culture No.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.
1	381	3.30	3.35	1.01	2.95	3.05	1.03	2.80	3.00	1.07	2.66	3.20	1.20
2	392	2.55	2.06	0.81	2.60	2.15	0.82	2.45	1.38	0.56	2.20	1.84	0.84
3	402	1.60	1.57	0.98	2.50	1.77	0.70	1.17	0	0	0.78	0.50	0.64
4	409	1.20	0.30	0.25	1.22	0.50	0.41	0			1.80	0.29	0.16
5	415	1.74	0	0	1.53	0	0				0	0	0
6	424	0			0								

		Experiment 5.			Experiment 6.			Experiment 7.		
		Relative increase.			Relative increase.			Relative increase.		
Passage No.	Culture No.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.
1	388	2.75	2.46	0.90	2.82	2.34	0.83	3.00	3.25	1.08
2	397	1.83	2.10	1.15	2.50	2.56	1.02	2.03	2.30	1.13
3	404	1.80	0.67	0.37	1.40	1.50	1.07	1.57	1.57	1.00
4	410	1.72	0	0	1.68	0.44	0.26	1.24	0.22	0.18
5	416	0			1.58	0.41	0.26	0.90	0.20	0.22
6	423				0	0	0	0	0	0

The tissues were incubated for 48 hours, and the rate of growth was measured in the ordinary manner.[4] Then they were transferred to a new medium of the same composition and the operation was repeated until death occurred, or until the rates of growth of fibroblasts in

[4] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

both experimental and control media had been compared during a number of passages.

1. Duration of Life of Fibroblasts in Media Containing 0 Per Cent and 7 Per Cent Serum.—Two groups of four experiments each were made. The results are averaged in Table I. The fragments of tissue in 0 per cent and 7 per cent serum grew at about the same rate. The fibroblasts migrated actively into the medium after each transfer. Nevertheless, the mass of the tissues never increased. After five or six passages, death occurred (Table I).

2. Duration of Life of Fibroblasts in Media Containing 0 Per Cent and 25 Per Cent Serum.—The first group was composed of four and the second of three experiments (Table II). The duration of life of the fibroblasts was slightly shorter in 25 per cent serum than in 0 per cent.

3. Duration of Life of Fibroblasts in Media Containing 50 Per Cent Serum.—The duration of life of fibroblasts in 50 per cent serum was compared to that in 25 per cent. In 25 per cent serum, death occurred after the fifth or sixth passage; in 50 per cent serum, after the fifth passage (Table III).

4. Rate of Growth of Fibroblasts in Media Containing 0 Per Cent and 10 Per Cent Serum in the Presence of 25 Per Cent Embryonic Tissue Juice.—The mass of the tissues increased, and the cultures had to be divided. But the rate of growth was no greater in 10 per cent serum than in the media containing no serum (Table IV). As a rule, the migration of the cells was slightly more active in the serum-free medium. As the life of fibroblasts in media containing embryonic tissue juice may be very long, and possibly indefinite, the condition of the tissues in both media was compared after ten passages. The average ratio of the rate of growth of fibroblasts in 0 per cent and 10 per cent serum was 0.96. There was, then, no difference between the tissues cultivated in media containing 0 per cent and 10 per cent serum, in the presence of embryonic tissue juice.

5. Rate of Growth of Fibroblasts in Media Containing 0 Per Cent and 25 Per Cent Serum in the Presence of 25 Per Cent Embryonic Tissue Juice.—The experiments summarized in Table V indicate that the presence of 25 per cent serum failed to increase the rate of growth of fibroblasts.

TABLE III.

Influence of 25 Per Cent and 50 Per Cent Serum on the Duration of Life of Homologous Fibroblasts.

Passage No.	Culture No.	Experiment 1. Relative increase. 25 per cent serum.	Experiment 1. Relative increase. 50 per cent serum.	Experiment 1. Ratio: $\frac{50 \text{ per cent}}{25 \text{ per cent}}$.	Experiment 2. Relative increase. 25 per cent serum.	Experiment 2. Relative increase. 50 per cent serum.	Experiment 2. Ratio: $\frac{50 \text{ per cent}}{25 \text{ per cent}}$.	Experiment 3. Relative increase. 25 per cent serum.	Experiment 3. Relative increase. 50 per cent serum.	Experiment 3. Ratio: $\frac{50 \text{ per cent}}{25 \text{ per cent}}$.
1	311	4.00	3.00	0.75	3.54	2.54	0.72	4.09	3.00	0.73
2	326	3.24	2.44	0.75	2.78	2.08	0.75	3.06	2.29	0.75
3	338	3.07	2.29	0.75	2.38	1.70	0.71	2.70	1.86	0.69
4	349	1.67	1.06	0.63	1.05	0.68	0.65	1.55	0.89	0.57
5	364	1.63	0.37	0.23	1.18	0	0	1.21	0.36	0.30
6	374	0.64	0		0			0	0	0

TABLE IV.

Influence of 0 Per Cent and 10 Per Cent Serum in the Presence of 25 Per Cent Tissue Juice on the Rate of Growth of Homologous Fibroblasts.

Passage No.	Culture No.	Experiment 1. Relative increase. Tyrode solution.	Experiment 1. Relative increase. Serum.	Experiment 1. Ratio: $\frac{S}{T}$.	Experiment 2. Relative increase. Tyrode solution.	Experiment 2. Relative increase. Serum.	Experiment 2. Ratio: $\frac{S}{T}$.	Experiment 3. Relative increase. Tyrode solution.	Experiment 3. Relative increase. Serum.	Experiment 3. Ratio: $\frac{S}{T}$.	Experiment 4. Relative increase. Tyrode solution.	Experiment 4. Relative increase. Serum.	Experiment 4. Ratio: $\frac{S}{T}$.
1	370	4.15	4.00	0.96	3.29	3.44	1.05	3.47	2.73	0.79	3.70	3.49	0.94
2	377	3.36	3.65	1.09	2.93	3.23	1.10	2.90	3.48	1.20	3.25	3.40	1.05
3	387	2.60	2.20	0.85	3.15	2.72	0.86	2.66	2.90	1.09	2.44	2.96	1.21
4	396	1.77	1.87	1.06	1.70	2.30	1.35	1.80	2.15	1.19	2.17	2.42	1.12
5	403	2.17	1.70	0.78	1.83	1.59	0.87	1.60	1.69	1.06	1.75	1.40	0.80
6	408	3.20	2.03	0.63	4.13	2.58	0.62	3.44	3.08	0.90	4.73	3.08	0.65
7	414	6.68	4.92	0.74	4.74	4.85	1.02	5.24	5.80	1.11	5.30	4.15	0.78
8	425	7.78	6.43	0.83	4.70	4.67	0.99	4.73	4.46	0.94	5.66	5.08	0.90
9	429	4.80	4.50	0.94	4.65	4.30	0.92	3.83	4.16	1.09	3.50	3.46	0.99
10	432	3.37	3.45	1.02	4.25	4.30	1.01	3.89	3.89	1.00	4.04	3.62	0.90

III.

DISCUSSION.

The duration of life of fibroblasts was found to be practically identical in media containing no serum and 7 per cent serum. It was slightly shortened by the addition of 25 per cent serum, and still more so by 50 per cent serum. Under the conditions of the experiments, serum proteins were apparently not utilized by the fibroblasts. These results confirmed the information obtained from previous experiments[1] in which the influence of serum on the activity of fibroblasts

TABLE V.

Influence of 0 Per Cent and 25 Per Cent Serum in the Presence of 25 Per Cent Tissue Juice on the Rate of Growth of Homologous Fibroblasts.

		Experiment 1.			Experiment 2.			Experiment 3.		
		Relative increase.			Relative increase.			Relative increase.		
Passage No.	Culture No.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.
1	433	3.10	3.24	1.05	3.14	3.27	1.04	3.82	3.54	0 93
2	444	4.23	4.50	1.06	4.40	4.52	1.03	4.00	4.30	1.08
3	454	3.46	3.25	0.94	3.35	3.34	1.00	4.32	3.65	0.84
4	466	3.45	2.90	0.84	3.85	3.85	1.00	4.50	4.05	0.90
5*	482	1.41	1.50	1.06	1.74	1.83	1.05	No measurement.		
6	491	3.54	3.43	0.97	4.40	4.35	0.99	4.56	4.56	1.00
7	498	4.15	3.80	0.92	4.15	4.28	1.03	4.15	4.63	1.12
8	513	2.95	3.10	1.05	No measurement.			No measurement.		
9	530	3.14	3.20	1.02	4.10	4.04	0.99	3.39	3.61	1.06

* Cultures divided, one-half discarded. Tracings made after 24 hours growth.

was studied during a short time. After ten passages in the presence of embryonic tissue juice, fibroblasts were as active in Tyrode solution alone as in Tyrode solution containing a small amount of serum. Their rate of growth was slightly decreased when serum was present in a higher concentration.

Serum, therefore, cannot be considered a culture medium in the proper sense of the word. No new protoplasm is built up by the cells from substances contained in the medium. The residual activity,

manifested by the cells in media composed only of plasma, serum, or Tyrode solution, is due to substances stored in the tissues or the cells themselves. The failure of the fibroblasts *in vitro* to utilize the serum proteins confirms the observation made by several experimenters[5] that tissues cultivated in plasma do not increase in mass, and that cell multiplication may be due to a mere transfer of food material from the central part of the tissue fragment to the periphery. As soon as embryonic tissue juice is added to the medium, the mass of the tissues increases rapidly. But the presence of serum under low or high concentration does not cause the production of a larger amount of tissue. The new protoplasm is made from substances contained in the embryonic tissue juice and not in the serum. Serum proteins are not used by the fibroblasts as food material. They appear merely to decrease the rate of cell multiplication.

IV.

CONCLUSIONS.

It may be concluded that, under the conditions of the experiments:

1. The duration of life of fibroblasts is not altered by the presence of 7 per cent serum in a medium composed of fibrin and Tyrode solution, but is slightly decreased when the concentration of the serum reaches 25 per cent.

2. Fibroblasts cultivated in serum or in Tyrode solution are only in a condition of survival; they do not build up new protoplasm from the serum proteins and their mass does not increase.

3. When embryonic tissue juice is added to the medium, the tissues increase in mass. But the rate of growth is the same in media containing 0 per cent and 10 per cent serum. In 25 per cent serum, however, the rate of growth slightly decreases. Even in the presence of embryonic tissue juice, serum does not increase the rate of growth of connective tissue.

4. The nitrogenous compounds contained in serum are not used as food material by fibroblasts growing *in vitro*.

[5] Lewis, M. R., and Lewis, W. H., *Anat. Rec.*, 1911, v, 277. Ingebrigtsen, R., *J. Exp. Med.*, 1912, xvi, 421. Burrows, M. T., *Anat. Rec.*, 1916–17, xi, 335. Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93.

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SURFACE TENSION OF SERUM.

VI. THE STUDY OF IMMUNE SERUM. TIME-DROP AND INITIAL VALUE OF SURFACE TENSION.

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PLATE 11.

(Received for publication, March 2, 1923.)

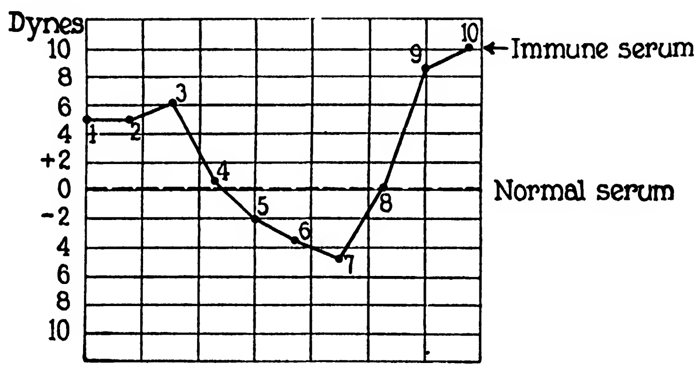
As stated in previous papers,[1-4] the four essential conditions under which the presence of antibodies in immune serum can be detected and followed by means of what might be termed the tensiometric method are: first, the dilution of the serum to 1:10,000; second, the use of strictly clean vessels; third, the care in handling the solutions, which must absolutely not be disturbed between the two measurements; and fourth, the study of the *time-drop*,[2] and not merely of the initial value of the surface tension. The first condition is a consequence of the numerous experiments reported in Papers I,[1] II,[2] IV,[3] and V.[4] The second has been emphasized in the same papers, and will be referred to again in the present paper. The third condition is satisfactorily fulfilled by the device represented in Fig. 1. However, the main object of this paper is to explain more extensively the reason of the fourth condition.

1. Time-Drop and Initial Value.—The results of a series of ten experiments on rabbits are reported in Text-fig. 1. From its mere inspection, it will be clear that the values of the initial surface tension of the solution of immune serum (1 part in volume of serum to 10,000 parts of saline 0.9 per cent) are sometimes higher and some-

[1] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575.
[2] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 707.
[3] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxvi, 547.
[4] du Noüy, P. L., *J. Exp. Med.*, 1923, xxxvii, 659.

times lower than the values of the solutions of normal serum. Hence, from these values alone, nothing could be gathered as to whether or not the serum contained antibodies. In order to make the chart clear, the initial value of the normal serum (before immunization) was taken as equal to 0, thus representing the axis of abscissæ, and the differences between the values of surface tension of the serum of the same animal, before and after immunization, were plotted with respect to this axis.

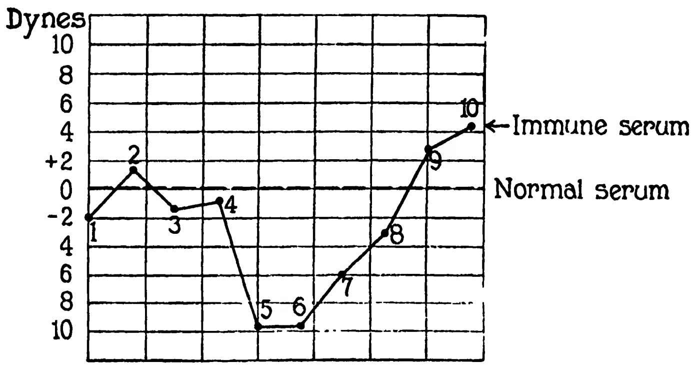
In Text-fig. 2, which represents the values of the surface tension of the solutions after standing for 2 hours, the same procedure was followed; *i.e.*, the values of the normal serum were taken as axis of 0,



TEXT-FIG. 1. Differences between the initial value of surface tension of normal and immune serum. Normal values are represented by the axis of abscissæ so that the differences are plotted in magnitude and sign.

and the differences between these values and those of the immune serum were plotted. For example, let us take two normal rabbits, A and B, whose serum solutions gave an initial value of surface tension of $A = 70.0$ dynes, and $B = 68.0$ dynes. After immunization, the initial value of the surface tension of the serum solution was $A_{imm.} = 73.0$ dynes, and $B_{imm.} = 66.0$ dynes. Then, if the normal values are used as the axis of abscissæ (ordinate = 0), the differences $A_{imm.} = +3.0$ dynes, and $B_{imm.} = -2.0$ dynes are plotted. In this way, the normal values will be represented by a straight line on both sides of which the differences between the values of the surface tension will be very obvious.

Consequently, if these two charts are brought together, however arbitrary the distance may be between the two parallel abscissæ representing the initial value of the normal serum, and the value after 2 hours, the ratio between their distance and the distance between the points representing the surface tension of immune serum in the two charts will express the ratio between the values of the so called time-drop in both cases, normal and immune serum. This was done in Text-fig. 3. It is clear that, in general, the variations in the value of the surface tension of immune serum after 2 hours



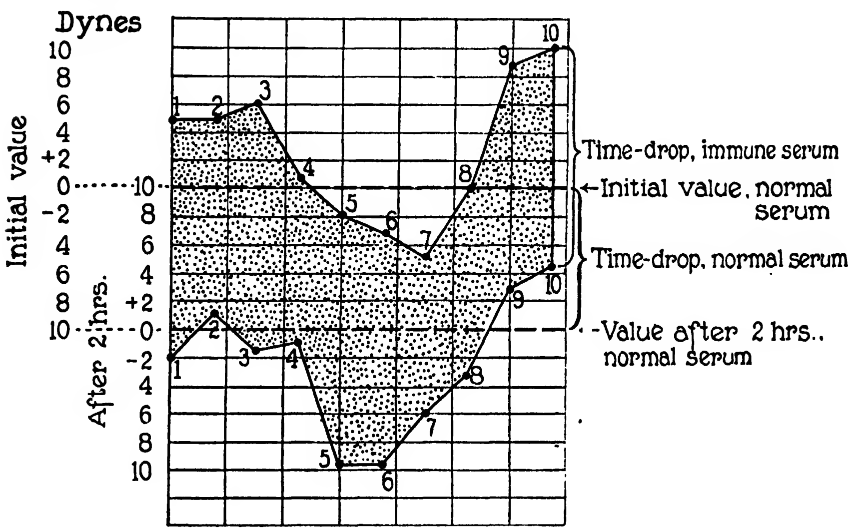
TEXT-FIG. 2. Differences between the values of surface tension of normal and immune serum after 2 hours. Normal values are represented by the axis of abscissæ so that the differences are plotted in magnitude and sign.

follow the fluctuations ot the initial value more or less closely, so that, whether they are higher or lower, the time-drop is always greater for immune serum.

By joining the values of the surface tension of immune serum, two broken curves are obtained. The area between them may be integrated mechanically (Text-fig. 3, stippled area). By comparing the area thus measured (which is proportional to the time-drop for immune serum) to the area comprised between the two parallel lines (which is proportional to the time-drop of normal serum), a ratio is obtained from which the mean increase per cent in the time-drop of serum after immunization can be computed. In the present chart (Text-fig. 3), the increase is approximately 45 per cent. Now, all these experiments were by no means perfect; in other

words, they include measurements which were not made at the time when the maximum drop occurred, due to the presence of antibodies; *i.e.*, 13 days. Some were made too soon, some too late. Only two of them were made at the exact time, and they show an increase of 78 per cent (Nos. 3 and 5). A previous experiment[4] even showed an increase of over 100 per cent.

2. Normal Time-Drop.—Since the publication of the first paper on the action of antibodies on the time-drop, a number of data have been gathered concerning the time-drop of normal rabbit serum.



TEXT-FIG. 3. Text-figs. 1 and 2 brought together. The stippled area represents the time-drop of immune serum, in other words the differences between the initial value and the value after 2 hours, and the white area comprised between the two parallel lines corresponds to the time-drop of normal serum.

Comparison of the figures showed that, in the instances studied at least, the general value of the time-drop of the serum solution of a healthy, normal rabbit varied between 5 and 8 dynes in 2 hours. A higher drop, let us say above 10 dynes, always indicated that the rabbit was not absolutely normal. In that case, the increase in time-drop after immunization was smaller. Later, it was found that an epidemic of snuffles had developed in the cages in which the animals were kept. Subsequently, in order to obtain constant

results, all animals whose time-drop was above 10 dynes were discarded as not absolutely normal.

3. Details of the Technique.—It is necessary to lay particular emphasis on the cleaning of *all* vessels in which the serum, its solutions, and the distilled water are to be contained. They should be boiled for 2 hours in a concentrated solution of sulfuric acid, to which have been added 15 cc. of a saturated solution of potassium dichromate per liter. Just as important are the freshness and purity of the distilled water, and the cleanness of the sodium chloride to be used. Ordinary C.P. NaCl should be washed in a separating funnel. After 24 hours, the solution is collected from the bottom without stirring, a large amount of the supernatant liquid being left in the funnel. The solution is then recrystallized and used, great care being taken to prevent dust particles from falling on the crystals. The watch-glasses should be washed only 2 or 3 days at most before they are to be used; otherwise the liquid will not wet the glass perfectly. All the pipettes while in the hot cleaning solution must be washed inside by aspirating the acid up and down many times, by means of a rubber bulb and tube.

If this method is followed, the surface tension of the saline solution may be checked over and over again and will always give the same reading, within 0.05 dyne; otherwise, the results will vary greatly.

As a test for the cleanness of the vessels and the purity of the water and salt, allow the pure NaCl solution to crystallize; the crystals should be similar to the pictures published in Paper II, and should leave no rings on the glass.

As the position of the maximum time-drop in function of concentration obviously depends on the size of the watch-glass, that is on the ratio $\frac{\text{Total surface of liquid}}{\text{Volume of liquid}}$, it is important always to use the same size watch-glass and the same amount of liquid; for maximum drop at 10^{-4}, diameter of free surface of liquid equals approximately 4 cm. and volume of liquid equals approximately 2 cc.

CONCLUSIONS.

1. The initial surface tension of serum or serum solutions is not affected systematically by the presence of antibodies in the serum.

On the contrary, the time-drop in 2 hours is always increased, from 25 to 100 per cent.

2. It is extremely important, in order to demonstrate these phenomena, that the greatest care be taken regarding the cleanness of the vessels and the purity of water and NaCl.

3. It is equally important to use a device, for example such as is pictured in this paper, capable of preventing the jarring and shaking of the liquid.

4. The value of the time-drop of a normal, healthy serum is never higher than 10 dynes. Should it be higher, the serum must not be used for immunity experiments.

EXPLANATION OF PLATE 11.

FIG. 1. Turntable on ball bearings used in connection with the tensiometer to prevent jarring of the solution. The watch-glasses are brought successively under the platinum loop and raised by means of a screw.

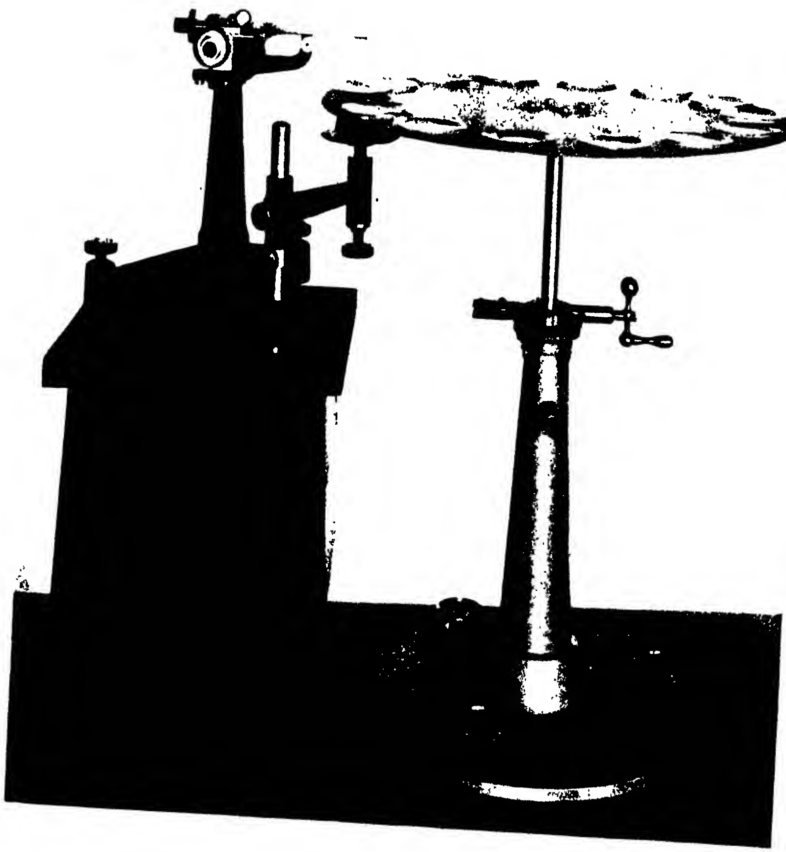


FIG. 1.

(du Noüy: Surface tension of serum. VI.)

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THE PHOTOCHEMICAL BASIS OF ANIMAL HELIOTROPISM.

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(Received for publication, February 15, 1923.)

I.

One of us published in 1888 a preliminary notice,[1] followed by a monograph in 1889,[2] in which it was shown by experiments chiefly on insects that the motion of animals to a source of light is not due to an attraction of the animals by the light, but is due to an automatic orientation of the animal by the light, as a consequence of which the animal is forced to move to the source of light. It was shown that if there is only one source of light, certain animals are automatically oriented by the light in such a way that their heads are turned towards the source of light and that their planes of symmetry are turned into the direction of the rays of light. In that case, the animal is automatically compelled to move to the source of light.

The action of light was ascribed by Loeb to a chemical effect on the retinæ or some sensitive spots of the skin. If there is only one source of light, and the plane of symmetry of the animal goes through the source of light, the symmetrical eyes (or the symmetrical photosensitive elements of the skin) are struck by the light at the same angle, and the intensity of illumination is the same for symmetrical elements. When, however, symmetrical elements of the eyes (or skin) of the animal are no longer struck at the same angle by the source of light, *e.g.* when the animal is illuminated sidewise, the intensity of illumination by the source of light is no longer the same for the symmetrical retinæ or other symmetrical photosensitive elements, and the animal is automatically compelled to change the direction of its motion in such a way that its plane of symmetry is again brought

[1] Loeb, J., *Sitzungsber. phys.-med. Ges. zu Würzburg*, 1888.

[2] Loeb, J., Der Heliotropismus der Tiere und seine Uebereinstimmung mit dem Heliotropismus der Pflanzen, Würzburg, 1889.

into the direction of the rays of light. As soon as this happens, the animal will be compelled again to move in a straight line towards the source of light. This was explained by Loeb in the following way. The tension of the symmetrical muscles of the locomotor organs is influenced by the light in a similar way to that in which it is influenced by the action of gravity on the internal ear in the higher animals. As long as the rate of photochemical change in symmetrical parts of the photosensitive organs is the same, the tension of symmetrical muscles in the locomotor organs—legs, wings, or swimmerets—will be affected in the same way and the animal will continue to move in as straight a line as the imperfections of its locomotor apparatus permit. When, however, the rate of photochemical change is no longer the same in symmetrical elements of the eye or skin, the tension of the symmetrical muscles of the locomotor apparatus will no longer be the same and the direction of the motion of the animal will be automatically changed. This change may either bring the head towards the source of light or away from the source of light. When the head is automatically turned towards the source of light, we speak of positive heliotropism, and when the head is turned automatically away from the light, we speak of negative heliotropism. As soon as the plane of symmetry falls again into the direction of the rays of light, the symmetrical muscles again assume the same tension and the animal is automatically forced to move in a straight line either towards or away from the source of light. What appeared to the earlier investigators as a mysterious attraction of the animals by the light (in the case of positively heliotropic animals) or as a mysterious flight from the light (in the case of negatively heliotropic animals) thus turned out to have been only a case of automatic orientation of the animal due to a photochemical effect on the retina or other photosensitive elements of the surface of the animal. The phenomenon was thus amenable to a purely physicochemical analysis, according to the principles of photochemistry.

II.

In an address delivered in 1911, Loeb suggested that the physicochemical law determining these automatic heliotropic reactions of animals was Bunsen and Roscoe's photochemical law, whereby the

photochemical effect, E, is proportional to the product of the intensity, I, into the duration, t, of illumination.

$$E = K \cdot I \times t$$

where K is a constant.[3] The idea was tested and confirmed in his laboratory by Ewald[4] on the heliotropic orientation of *Daphnia*, by Loeb and Ewald,[5] by Loeb and Wasteneys[6] on the heliotropic curvature of *Eudendrium*, by Loeb and Northrop[7] on the larvæ of *Balanus*, and by Patten,[8] in Parker's laboratory, on the heliotropic orientation of the larvæ of the blowfly.[9] It is intended to show in this paper that the law holds also for the orientation of the horseshoe crab (*Limulus*).

Young specimens of the horseshoe crab about 15 cm. in length were used. In each experiment one specimen was put into a square aquarium with straight glass walls (Fig. 1), and its orientation under the simultaneous influence of two lights, *a* and *b*, was ascertained. In order to permit the exact measurement of the orientation of the animal with respect to the lights, it was necessary to limit the extent of motion of the animal, without limiting its freedom of assuming a definite orientation with regard to the two lights. For this purpose the tail of the animal was fastened with the loop of a short cotton thread to a nail fixed in the bottom of the aquarium so that the animal could turn without friction in any direction without being able to move beyond the distance of the length of the thread from the nail. Loeb had found that the larvæ of *Limulus* are positively heliotropic in cold, and negatively heliotropic in warmer water.[10] This seems to be the case for these older specimens too, which were mostly negative at room temperature, though not all the specimens reacted to light. A small number, ten out of forty-eight specimens, could be used for the tests to be described. That not all reacted may or may not have been due

[3] Loeb, J., The mechanistic conception of life, Chicago, 1912.

[4] Ewald, W. F., *Science*, 1913, xxxviii, 236; *Z. Sinnesphysiol.*, 1914, xlviii, 285.

[5] Loeb, J., and Ewald, W. F., *Zentr. Physiol.*, 1913–14, xxvii, 1165.

[6] Loeb, J., and Wasteneys, H., *J. Exp. Zool.*, 1917, xxii, 187.

[7] Loeb, J., and Northrop, J. H., *Proc. Nat. Acad. Sc.*, 1917, iii, 539.

[8] Patten, B. M., *J. Exp. Zool.*, 1914, xvii, 213.

[9] All the work on Loeb's theory of tropisms is discussed in his book, Forced movements, tropisms, and animal conduct, Philadelphia and London, 1918.

[10] Loeb, J., *Arch. ges. Physiol.*, 1893, liv, 81.

to their exhausted condition, since they had been kept in the laboratory under unfavorable conditions.

Two Mazda incandescent lamps of equal intensity (determined with the Lummer-Brodhun photometer) were used. They were placed as indicated in Fig. 1. In order to allow the exact measurement of the

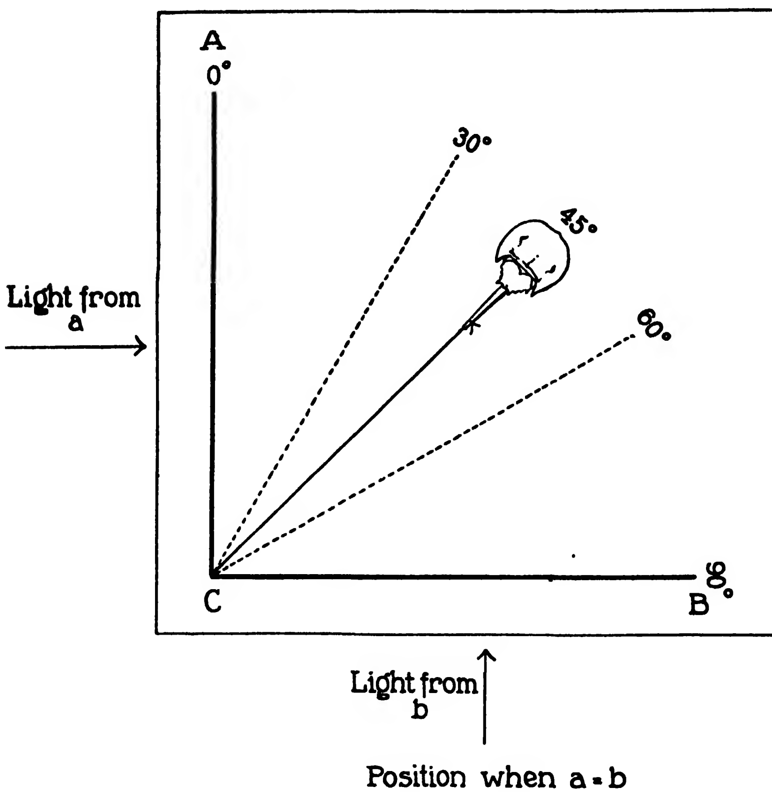

FIG. 1. Orientation of the negatively heliotropic crab with respect to two lights, *a* and *b*, when the intensity of the two lights is equal.

orientation of the plane of symmetry of the animal with respect to the two lights, a quadrant, with the nail as center, marked on the bottom of the aquarium, was divided into sectors of 5° each, the zero line being parallel to one illuminated side of the aquarium, the 90° line to the other illuminated side (Figs. 1 and 2).

When only one light was used, *e.g.* light *a*, the negatively heliotropic animal oriented itself in the path of the light, namely the line CB (90°), with its tail toward the light; when only light *b* was turned on, the animal came into the stationary position of 0°. In each case the animal put its plane of symmetry into the direction of the rays of

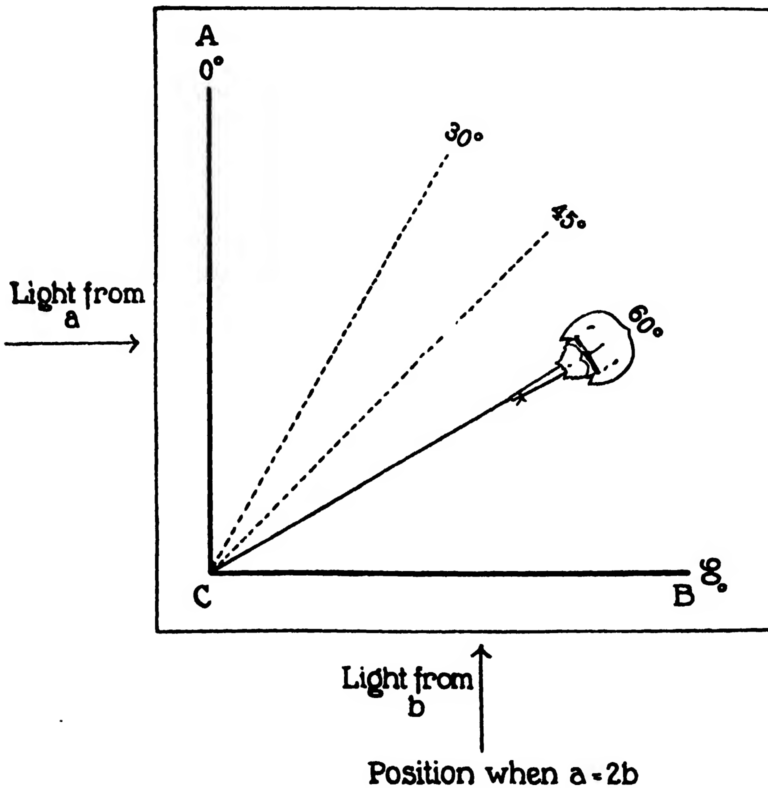

FIG. 2. Orientation of the negatively heliotropic crab with respect to two lights, *a* and *b*, when the intensity of *a* is twice as great as the intensity of *b*.

light. When both lights were turned on simultaneously and when the intensity of the two lights was equal, the plane of symmetry of the animal coincided with the angle of deflection of 45° from the line AC (Fig. 1). During the whole time the animal made active swimming motions, trying to escape, and these motions kept it in the stationary position of about 45°, as shown in Fig. 1.

In all the following experiments the intensity of light *a* remained stationary, namely 150 meter candles, while the intensity of light *b* was changed by increasing its distance from the aquarium.

When light *b* was put so far away from the aquarium that its intensity was only 75 meter candles, *i.e.* only half the intensity of *a*, the orientation of the animal was different. It put itself into the position indicated in Fig. 2, so that its plane of symmetry formed no longer an angle of 45° but on the average one of about 60° with AC. This was presumably the angle at which the intensity of illumination of the left and right retinæ was equal according to the cosine law to be discussed later.

When the intensity of light *b* was reduced to 37.5 meter candles, while that of light *a* remained constant (namely 150 meter candles), the animal oriented itself in such a way that its plane of symmetry formed on the average an angle of about 68° with the line CA.

III.

In the experiments thus far mentioned only the intensity of illumination of the two lights *a* and *b* varied, while the duration of illumination was the same for both lights. The duration of illumination of the animal by light *b* can be reduced by putting a rapidly rotating opaque disc (made of thick cardboard), with one sector cut out, between the light and the aquarium.[11] If a sector of 180° is cut out from the rotating disc, light from *b* will reach the animal only during each half period of rotation of the disc, and the duration of illumination of the animal by this light will be reduced to one-half. If a sector of 90° is cut out from this opaque disc and put between light *b* and the animal, the duration of illumination of the animal by light *b* is reduced to one-fourth. Now if the general law of photochemistry, namely the Bunsen-Roscoe law, determines the heliotropic orientation of the animal, the animal should always put its plane of symmetry into the line bisecting the angle ACB, whenever the product of intensity into duration of illumination is the same for the two lights *a* and *b*.

[11] This method of varying the duration of illumination had been used by Ewald, Patten, and Loeb and Northrop in the papers referred to.

In all the following experiments the intensity of light *a* was kept constant, namely 150 meter candles, while the intensity and duration of illumination of *b* were changed.

When the intensity of light *b* was increased to 300 meter candles, while the intensity of light *a* remained 150 meter candles, it was found that the animal put its plane of symmetry into the line 45° (Fig. 1), bisecting angle ACB, when the open sector of the rotating disc was 180°. In this case the duration of illumination by light *b* was cut into two, so that the product of duration into intensity of illumination was for light *a*

$$150 \times t$$

and for light *b*

$$300 \times \frac{t}{2} = 150 \times t$$

In other words, the orientation of the animal was determined by the Bunsen-Roscoe law. When the intensity of light *b* was raised to 600 meter candles, while light *a* remained 150 meter candles, it was found necessary to reduce the open sector of the rotating disc between light *b* and the animal to 90°, to force the animal to put itself in the position of Fig. 1, where its plane of symmetry bisected the angle ACB. In this case the product of duration into intensity of illumination was again the same for the two lights, namely for light *a*

$$150 \times t$$

and for light *b*

$$600 \times \frac{t}{4}$$

which again conforms with the Bunsen-Roscoe law.

IV.

We have seen that when no rotating disc is used, *i.e.* when the duration of illumination is the same for both lights, the animal puts its plane of symmetry into an average angle of about 60° with the line AC when the ratio of the intensity of *a* to *b* is as 2:1. Now the law of Bunsen and Roscoe demands that this orientation should always occur when the ratio, product of $I \times t$ for light *a* over that of the

same product for light *b*, is as 2: 1, regardless of whether the intensity alone or the duration alone or both are changed. This was found to be the case.

When the intensity of both lights was 150 meter candles, the duration of illumination of light *b* had to be cut exactly to one-half with the aid of the rotating disc to force the crab to put its plane of symmetry into the position shown in Fig. 2, where the plane of symmetry of the animal forms an angle of 60° with the line AC.

In another series of experiments the intensity of light *b* was increased to 300 meter candles, while that of light *a* remained 150 meter candles. In this case it was found necessary to cut down the time of illumination of light *b* to one-fourth (by using a rapidly rotating disc with a sector of 90° cut out) in order to compel the animal to put its median plane at an angle of 60° with the line AC. In both of these experiments the value of the illumination product, $I \times t$, for *a* and *b*, was as 2: 1.

Finally it was possible to show that in order to compel the animal to put its plane of symmetry into a line forming an angle of about 68° with the line AC, it was necessary to make the value of the product $I \times t$ for light *b* one-fourth of the value of the product $I \times t$ for light *a*. Thus light *a* had the intensity of 150 meter candles and no disc was put before this light. When the light *b* had the intensity of 75 meter candles, the open sector of the rotating disc between light *b* and the animal had to be 180° in order to force its plane of symmetry into forming an angle of 68° with the line AC. In this case the ratio of the illumination products $I \times t$ for the two lights *a* and *b* was

$$Ia \times ta : Ib \times tb = 150 \times 1 : 75 \times \tfrac{1}{2} = 4 : 1$$

When the intensity of light *b* was 150 meter candles, the open sector of the rotating disc had to be 90° to compel the animal to put its plane of symmetry into a line forming an angle of 68° with AC. In this case the ratio of illumination product for *a* and *b* was again 4: 1.

$$Ia \times ta : Ib \times tb = 150 \times 1 : 150 \times \tfrac{1}{4} = 4 : 1$$

These results leave no doubt that the heliotropic orientation of the horseshoe crab is determined by a photochemical reaction which follows the law of Bunsen and Roscoe.

V.

The results of our experiments are tabulated in Table I. For the explanation of this table the following data should be remembered. The intensity of light *a* (*Ia*) was always constant, namely 150 meter candles, and so was the duration of illumination *t* of light *a*, which was 1, since no rotating disc was put in front of this light. The illumination product for light *a*, $Ia \times ta$, was therefore always $150 \times t$. The intensity of light *b* (*Ib*) varied, as the first vertical column of the table indicates, between 0 and 600 meter candles. The relative duration of illumination, *tb*, varied between 0.25 and 1.0. This time is the fraction of the period of rotation during which the light could reach the animal.

The third column of the table gives the illumination product for light *b*, namely $Ib \times tb$, and the fourth column gives the ratio of the illumination products of light *b* and light *a*, namely

$$\frac{Ib \times tb}{Ia \times ta}$$

The last vertical row of the table gives the average angle of the plane of symmetry of the animal with the line AC (Figs. 1 and 2).

The table shows that the value of this angle is the same for the same value of

$$\frac{Ib \times tb}{Ia \times ta}$$

In order to give the reader an idea of the individual variations, the value of the angle of orientation of the animal is given for each of the individual specimens of *Limulus* tried.

Ten individuals were found to orient themselves to the light, practically all being negatively heliotropic. Each animal is numbered and some animals were used repeatedly on different days; *e.g.*, Animals 1, 6, and 9.

Each individual figure is the average of ten consecutive measurements taken within 1 minute. The animal made constant swimming motions but was prevented from making any progressive motion, being held by the thread attached to the nail (Figs. 1 and 2).

All the variations in intensity and duration of illumination of light *b* recorded in the vertical Column 1 were tried for each animal in rapid

succession. There were variations in the orientation of different individuals but these were chance variations which were equal and opposite for different individuals. The average angular deflection of the animal from the path of light *b* (the line AC) is given in the last vertical column of Table I.

TABLE I.

Angular Deflection of Limulus from Path AC of Light b.

Intensity of light *a* constant = 150 meter candles.
Duration of illumination by light *a* constant = 1.
Intensity × duration of light *a* constant = 150.
Intensity and duration for light *b* variable.

I_b	t_b	I_bt_b	Ratio $\frac{I_bt_b}{I_at_a}$	7	5	2	3	4	1	1	6	6	12	9	9	9	18	Average angular deflection.
meter candles																		
150	1.0	150	1.0	40	46.5	45	46	46.5	46	47	50	42	50	43	41	43	42	45.1 ±0.6
300	0.5	150	1.0	45	42		44	42.5	43	48	57	41	43	41	43	46	49	45.2 ±0.8
600	0.25	150	1.0	45	45	42	44	44.5	48	50	52	40	49	46	46	46	42	45.7 ±1
75	1.0	75	0.5	60	55	65	70	69	72	54	70	46	58	58	53	56	50	59.7 ±2
150	0.5	75	0.5	61	60	59	71	70	69	58	63	55	62	63	58	57	51	61.2 ±2
300	0.25	75	0.5	60	58		68	69	68	56	61	53	50	63	64	58	51	60.0 ±2
37.5	1.0	37.5	0.25	70	61	70	74	78	79	66	78	61	71	64	67	69	63	68 ±2
75	0.5	37.5	0.25	67	63		70	73	72	59	75	57	70	67	68	60	57	66 ±2
150	0.25	37.5	0.25	70	60	64		74	79	65	78	66	71	69	70	68	53	68 ±2
0	1.0	0	0	89.5	88	92	91	90	88	87	92	86	91	91	90	87		89.4 ±0.6

VI.

An animal is therefore automatically oriented by the light in such a way that the illumination products $I \times t$ are the same for the two eyes. When only one source of light exists, this is always the case when the plane of symmetry of the animal falls into the direction of the rays of light, *i.e.* goes through the source of light. When two lights of equal intensity act simultaneously on the animal, this happens when the plane of symmetry of the animal bisects the angle that its head (or rather that point of its head which we may assume to represent Hering's imaginary cyclopic eye, substituted for the retinæ of the animal) forms with the two lights.

In both cases the symmetrical points of the eyes or skin of the animals are struck by the rays of light at the same angle. The importance of this angle was pointed out by Loeb in his first publications referred to above. The photochemical significance of this angle lies in the cosine law which states that the intensity of illumination of a surface element varies with the cosine of the angle of incidence of the light. If this angle be α, then the expression for the orienting effect, E, of light becomes

$$E = K \cdot I \times t \times \cos \alpha$$

Now Loeb had also insisted on the fact that symmetrical elements of the eyes or of the photosensitive parts of the skin of the animal are not only equal morphologically but also chemically; *i.e.*, the same illumination will produce equal chemical effects on two symmetrical elements, but not, as a rule, on dissymmetrical elements. This explains why, when only one source of light exists, the animal is automatically oriented by the light in such a way that symmetrical elements of the retinæ are struck by the light at the same angle, α. In this case the rate of photochemical change in both eyes is the same when I and t are the same. The heliotropic orientation of animals by light is, therefore, such that the value of the product

$$I \times t \times \cos \alpha$$

is the same for both eyes (or any other symmetrical photosensitive elements of the animal).

It is obvious what must happen when the value of $I \times t$ is no longer the same in both eyes. If α remains the same for both eyes, the rate of photochemical change in the two retinæ will be different when $I \times t$ is different for the two eyes, and hence the symmetrical muscles of the locomotor organs will undergo a different degree of tension, a fact to which we shall return presently. If the value of $I \times t$ is greater in the left than in the right eye (as in Fig. 2), the equality of photochemical effect in the two eyes can be restored only when the value of cos α becomes greater for the right eye (b) than for the left eye (a), $\cos \alpha_b > \cos \alpha_a$ (Fig. 2), or, in other words, the animal will be automatically compelled to put its plane of symmetry into such a position that it forms a greater angle than 45° with the line AC which

is nearer the stronger light *a*. Through this change of position α becomes smaller in the right than in the left eye and hence cos α becomes greater in the right than in the left eye. This is rendered evident in Diagram 3. It would be even possible to calculate beforehand the ratio of the two angles for each case if only the surfaces of the retinæ were plane and parallel. According to Loeb's theory the animal must put its plane of symmetry into such a position that the product of

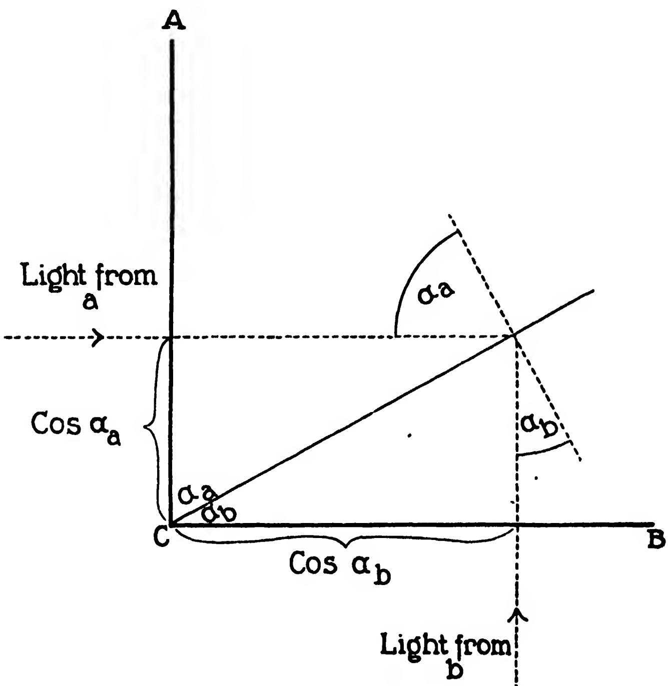

FIG. 3. Diagram illustrating the variations of the intensity of illumination with the cosine of the angle of incidence.

$I \times t \times \cos \alpha$ is the same for the two eyes. If now $I \times t$ is twice as great for the left as for the right eye,

$$2 \times \cos \alpha_a \text{ (left eye)} = \cos \alpha_b \text{ (right eye)}$$

In other words, the cosine of the angle of incidence of light on the left retina, cos α_a, must be only one-half of the cosine of the angle of incidence in the right eye, cos α_b. The animal must change its position

in such a way that the angle of incidence, α_a, for the stronger light on the left retina is greater than the angle of incidence, α_b, for the weaker light on the right retina (Fig. 3), a result which is actually observed. On account of the curvature of the retinæ of the horseshoe crab and on account of the fact that it possesses in addition to two lateral, also median eyes, it is impossible to test this postulate of Loeb's theory quantitatively for *Limulus*. Patten's[8] observations on the heliotropic orientation of the larvæ of the blowfly may be used as an approximate quantitative confirmation of this part of the theory. Qualitatively, however, the theory is supported by all the observations of the heliotropic reactions of animals.

VII.

Finally the question arises as to why it happens that the animal is automatically oriented in such a way that $I \times t \times \cos \alpha$ has the same value for the symmetrical eyes or the symmetrical elements of its photosensitive surface. The answer to this was given by Loeb by the suggestion that the products of decomposition of the photosensitive substance act on the sensory nerves connected with the eye and thereby influence the tension of the muscles. The connection between the rate of photochemical change and muscular reaction of the animal has been investigated by Hecht[12] from a physicochemical viewpoint in the case of reactions of *Mya* and *Ciona* to light, and he has been able to define the nature of the reaction. On the basis of his measurements it is safe to say that the rate of decomposition of a photosensitive substance in the eye or skin of an animal influences the tension of muscles. Now experiments on the influence of gravity on the ear of higher animals leave no doubt that the tension of the symmetrical groups of muscles which move the eyes or head and subsequently the whole body to the right or left is acted upon as a unit; and also that these two units are linked with the two symmetrical halves of the photosensitive or the geotropically sensitive elements of eyes or internal ear respectively. Experiments have shown that upon asymmetrical illumination animals are either compelled to move to that side where the product $I \times t \times \cos \alpha$ is greater or where it is smaller;

[12] Hecht, S., *J. Gen. Physiol.*, 1919–20, ii, 229, 337; 1920–21, iii, 367, 375.

in the former case we speak of positively heliotropic animals, in the latter of negatively heliotropic animals. In the case of positively heliotropic animals the tension of muscles turning the head towards that source of light increases with the value of $I \times t \times \cos \alpha$, while in the case of negatively heliotropic animals the tension of the same muscles is lowered. The reason for this difference is not yet known. When the eyes of a positively heliotropic animal are struck asymmetrically by light the rudder action of the swimmerets, turning the head toward the source of light, will be stronger than that of their symmetrical antagonists, and the animal will automatically deviate towards the source of light until its plane of symmetry is again in such a position that the value of $I \times t \times \cos \alpha$ is again the same for both eyes. In that case the influence of the light on symmetrical muscles is the same and the animal will continue to move in that direction. This part of Loeb's theory has been put to a test by a number of writers, among others Holmes,[13] Garrey,[14] Minnich,[15] and Cole,[16] and proved to be correct. Of the many experiments of these authors special attention may be called to the fact that when one eye is covered, the animal moves constantly in a circle around a source of light, the open eye facing the center of the circle when the animal is positively heliotropic. This is a consequence of the fact that in such a case the rudder action of the symmetrical organs of locomotion is no longer the same but is stronger in those muscles which turn the head of the animal towards the source of light. Moreover, it was shown by Garrey[14] and Cole[16] that the curvature of the circle in which such animals move becomes the greater the greater the intensity of light. Cole[16] has shown that the relation between intensity of light and curvature is logarithmic in agreement with the Weber-Fechner law. It follows further that the heliotropic orientation can only be expected in a moving animal, since when the animal rests there can only be a difference in the relative tension of symmetrical muscles. This may result in peculiar postures which have been observed by Garrey,[14] but it is not necessary that they should result in a definite orientation

[13] Holmes, S. J., *J. Comp. Neur. and Psych.*, 1910, xx, 145.
[14] Garrey, W. E., *J. Gen. Physiol.*, 1918–19, i, 101.
[15] Minnich, D. E., *J. Exp. Zool.*, 1919, xxix, 343.
[16] Cole, W. H., *J. Gen. Physiol.*, 1922–23, v, 417.

of the plane of symmetry with reference to the light. Thus heliotropic animals at rest may occupy any position with reference to the light, while when they move they will be automatically oriented on account of the rudder effect of the symmetrical legs or swimmerets of the animal.

Differences in the heliotropic sensitiveness of different animals will probably be found to depend upon the relative mass and nature of the photosensitive substance in their eyes or skin, in the relative quantity of decomposition products required to cause reflexly a change in the tension of their muscles, and in the nature of the nervous connection of the eyes with the symmetrical muscles of locomotion.

VIII.

SUMMARY.

1. Experiments on the heliotropic orientation of *Limulus* were made which confirmed Loeb's photochemical theory of animal heliotropism proposed first in 1888 and 1889 in experiments on insects, and later in experiments on other forms of animals.

2. It is shown that these animals are oriented by light in such a way that the product

$$I \times t \times \cos \alpha$$

is the same for the symmetrical photosensitive elements of the eyes or the skin, where I is the intensity of the light, t the duration of illumination, and α the angle of incidence of the light at the surface element of the photosensitive organ.

3. When this equation holds, the products of decomposition by light must be the same in symmetrical elements of the eyes or skin, and the influence of these products of decomposition on the tension of symmetrical muscles of the locomotor organs of the animal must be the same. As a consequence the animal must move in the path of light, either to or from the source of light.

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THE STABILITY OF BACTERIAL SUSPENSIONS.

VI. THE INFLUENCE OF THE CONCENTRATION OF THE SUSPENSION ON THE CONCENTRATION OF SALT REQUIRED TO CAUSE COMPLETE AGGLUTINATION.

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It has been shown in the previous papers of this series[1] that the addition of electrolytes to suspensions of bacteria (*Bacillus typhosus* and the bacillus of rabbit septicemia) affects two properties of the suspension. First, the potential difference between the organisms and the liquid is changed, and second, the cohesive force (the force with which the organisms adhere) is affected. Low concentrations of electrolytes affect primarily the potential, and higher concentrations (more than 0.1 N) primarily the cohesive force. It has further been shown that, if the cohesive force is not affected, agglutination occurs whenever the potential is reduced below about 13 millivolts. If the cohesive force is decreased, this critical potential is lowered and in high concentration of salt no agglutination occurs, although the potential may be too small to measure. In the case of suspensions which had been treated with immune serum, the cohesive force is not affected by any concentration of salt, and agglutination occurs whenever the potential is decreased to less than 13 millivolts. Suspensions sensitized with immune serum therefore behave just as oil emulsions studied by Powis[2] and present a much less complicated phenomenon. The point of complete agglutination in this case is therefore a convenient method of determining the salt concentration required to decrease the potential to 13 millivolts.

[1] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 629. Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639, 655. De Kruif, P. H., and Northrop, J. H., *J. Gen. Physiol.*, 1922–23, v, 127, 139.

[2] Powis, F., *Z. physik. Chem.*, 1915, lxxxix, 186.

EXPERIMENTAL PROCEDURE.

Suspensions of *Bacillus typhosus* were used. They were prepared by washing in distilled water as described in a previous paper,[1] and then suspended in $\frac{1}{20}$ of the volume of the original broth culture. 0.5 cc. of a powerful antityphoid horse serum was then added to 500 cc. of this suspension and dilutions were made in distilled water so as to furnish suspensions of relative concentrations of 1, 3, and 9. The amount of serum added was in excess of that needed to cause agglutination with the minimum amount of NaCl. The amount of electrolyte added with the serum is negligible.

1 cc. of the suspensions was then added to 1 cc. of the salt solutions as noted in the tables, the tubes were allowed to stand at 20°C. for 18 hours, and the degree of agglutination was read. The sharpest point is that marked as complete (C). This represents the condition in which the suspension is completely settled and the supernatant liquid perfectly clear. +++ was recorded when the supernatant liquid was slightly cloudy.

The results of the experiments are shown in Tables I and II. Table I shows that the concentration of NaCl, $NaNO_3$, Na_2SO_4, KCl, LiCl, NaBr, NH_4Cl, $BaCl_2$, and $MgSO_4$ required to cause complete agglutination is the same irrespective of the concentration of the suspension. The potential measurements reported in a preceding paper show that none of these salts reverses the sign of the charge. The results of the different salts are not comparable as they were made at different times and in some cases with different suspensions. The result of importance here is the comparison of the degree of agglutination of the various concentrations of suspension with the same salt. The series with any one salt were made at the same time and under as closely comparable conditions as possible.

The results of the experiments with those salts that had been found to reverse the sign of the charge on the organism (except for $ZnSO_4$) are given in Table II. The table shows that in each case the amount of salt increases in proportion to the concentration of suspension, as nearly as can be determined from the experimental data. The results with $LaCl_3$ are especially interesting, since there are two zones of agglutination. The one in the lower concentration represents the

TABLE I.

Effect of Concentration of Sensitized Typhoid Suspension on Concentration of Salt Required to Cause Complete Agglutination.

Salt.	Relative concentration of suspension.	Degree of agglutination in the following concentration of salts; equivalents per liter.					
		0.05	0.025	0.0125	0.00625	0.0031	0.0015
NaCl	1	C.	C.	+++	++	+	+
	3	C.	C.	+++	++	+	+
	9	C.	C.	+++	+++	++	+
$NaNO_3$	1	C.	C.	+++	++	+	–
	3	C.	C.	+++	++	+	+
	9	C.	C.	+++	++	–	+
Na_2SO_4	1	C.	C.	+++	++	+	–
	3	C.	C.	+++	++	+	+
	9	+++	C.	C.	++	+	+
KCl	1	C.	C.	+++	++	++	+
	3	C.	C.	+++	++	++	+
	9	C.	+++	+++	+++	++	+
LiCl	1	C.	C.	C.	++	+	+
	3	C.	C.	C.	++	+	+
	9	C.	C.	C.	++	+	+
NaBr	1	C.	C.	C.	++	+	+
	3	C.	C.	C.	++	+	+
	9	C.	C.	C.	++	+	+
NH_4Cl	1	C.	C.	+++	++	+	+
	3	C.	C.	+++	++	+	+
	9	+++	C.	+++	++	+	+
$BaCl_2$	1	C.	C.	C.	+++	++	+
	3	C.	C.	C.	+++	++	+
	9	C.	C.	C.	+++	++	+
$MgSO_4$	1	C.	C.	C.	+++	++	+
	3	C.	C.	C.	+++	++	+
	9	C.	C.	C.	+++	++	+

point at which the sign of the charge changes. The agglutination in high concentration is at the point at which the charge is again lowered, but does not become reversed.

TABLE II.

Effect of Concentration of Sensitized Typhoid Suspension on the Concentration of Salt Required to Agglutinate.

Electrolyte.	Concentration of suspension.	Degree of agglutination in the following concentrations of salts; equivalents per liter.														
		0.51	0.256	0.128	6.4×10^{-2}	3.2×10^{-2}	1.6×10^{-2}	8×10^{-3}	4×10^{-3}	2×10^{-3}	1×10^{-3}	5×10^{-4}	2.5×10^{-4}	1.28×10^{-4}	6.4×10^{-5}	3.2×10^{-5}
$HgCl_2$	1						C.	C.	C.	C.	C.	C.	+++	++		
	3						C.	C.	C.	C.	C.	+++	++	+		
	9						C.	C.	C.	C.	+++	++	+	−		
$ZnSO_4$	1						C.	C.	C.	C.	+++	+	+			
	3						C.	C.	+++	+++	++	+	−			
	9						C.	+++	++	++	+	+	−			
Copper acetate	1			+++	++	+	−	−	+	+++	C.	C.	C.	++	+	−
	3			++	+	+	+	++	C.	C.	C.	+++	++	+	+	−
	9			++	+	++	+++	C.	C.	C.	+++	++	++	+	+	−
$LaCl_3$	1	C.	C.	+++	++	+	+	−	−	−	+	+	C.	+	−	
	3	C.	C.	+++	++	+	+	−	+	+++	C.	+++	+	+	−	
	9	C.	C.	+++	++	++	++	+	C.	+++	+++	+	+	+	−	
HCl	1							−	−	−	−	+	+	C.	−	−
	3							−	−	−	+	C.	+	−	−	−
	9							−	−	++	C.	+	+	−	−	−
H_2SO_4	1							−	−	−	+	C.	+	−		
	3							−	−	+	C.	+	−	−		
	9							+	+	C.	+	−	−	−		

It will be noted that there is no distinct valency effect as far as the relation of the suspension concentration to the agglutinating concentration of salt is concerned.

The influence of the chemical nature of the ion, however, is apparent. The alkali and alkali earths, which are inert as regards proteins and do not form complex ions easily with ammonia or amino groups, do not combine chemically or reverse the sign of the organisms. The heavy metals and hydrogen ion, which are known to form complex ions readily with ammonia and amino groups in general, do reverse the sign of charge and behave as though they combined chemically with the suspension.

SUMMARY.

1. The concentrations of various salts required to agglutinate different concentrations for a suspension of typhoid bacilli sensitized with immune serum have been determined.

2. The electrolytes may be divided into two classes; (1) those with which the concentration required to agglutinate is independent of the concentration of the suspension; and (2) those with which the agglutinating concentration increases in proportion to the concentration of the suspension.

3. The salts comprised under (1) do not reverse the sign of the charge of the suspension.

4. The salts of Class (2) (with the exception of $ZnSO_4$) do reverse the sign of the charge.

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MEMBRANE POTENTIALS IN THE DONNAN EQUILIBRIUM.

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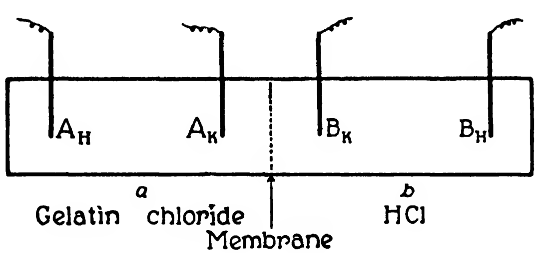
The development of Loeb's theory of the colloidal behavior of the proteins has recently been criticized by A. V. Hill.[1] This criticism seems to be based on a misunderstanding, which it is hoped the following considerations may help to clear up.

The arrangement of the experiments in question is quite clearly indicated by the diagram given by Hill, which is reproduced herewith.



(The diagram gives, however, no indication of the hydrostatic pressure due to the liquid in the manometer tube, which is a necessary condition for equilibrium. For further details of the experiments Loeb's original papers and book[2] may be consulted.) A_H and B_H represent hydrogen electrodes; A_K and B_K, calomel electrodes connected to the solutions by saturated KCl.

Loeb measured the potential difference between A_K and B_K, which he termed the observed P.D., or membrane potential. He also meas-

[1] Hill, A. V., *Proc. Roy. Soc. London*, Series A, 1923, cii, 705.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

ured the pH of each solution separately; that is, he measured the P.D. between A_H and A_K and that between B_H and B_K. The difference between these values he termed, somewhat unfortunately, perhaps, the calculated P.D. This term is being replaced in his more recent writings by the term hydrogen electrode potential. The agreement between the membrane potential and the hydrogen electrode potential was interpreted by Loeb to indicate the applicability of Donnan's theory of membrane equilibria.

Donnan had predicted that when two solutions were separated by a membrane impermeable to one ion of one of the solutions, but permeable to other ions, then, at equilibrium, the product of the concentrations of the diffusible ions on one side of the membrane should be equal to the product of the corresponding concentrations on the other side. He further predicted that a potential difference should exist between the two solutions, and that its magnitude should be given by the relation

$$E = \frac{RT}{F} \ln \frac{x}{y}$$

where x and y represent the concentrations of any one of the diffusible ions in the two solutions, and the other symbols have their usual meaning. These relations were deduced by Donnan on the basis of the laws of thermodynamics, as applied to the equilibrium in a system of the type already defined.

Hill maintains that this agreement of the quantities which Loeb has termed membrane potential and hydrogen electrode potential "is a necessary consequence of *any* mechanism which does not offend the Second Law of Thermodynamics. . . . " This is quite true; but in this case it is just this particular mechanism of a membrane impermeable only to gelatin which has brought about the establishment of equilibrium. It had been shown repeatedly that the collodion membranes used in Loeb's experiments were impermeable to proteins but permeable to simple inorganic electrolytes. The existence of protein ions in these solutions was shown by the earlier experiments[8]

[8] Hardy, W. B., *J. Physiol.*, 1905–06, xxxiii, 251.
Pauli, W., Kolloidchemie der Eiweisskörper, Dresden and Leipsic, 1920.
Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914 and 1922.
Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1917, xii.
Robertson, T. B., The physical chemistry of the proteins, New York and London, 1918.

of Hardy, Pauli, Michaelis, Sörensen, Robertson, Loeb,[2] and others, on electrical migration and electrometric titration of protein solutions. Hence the conditions necessary for the establishment of a Donnan equilibrium were known to be satisfied; Loeb's experiments tested and verified this idea.

Hill's criticism takes no account of the fact that Loeb's experimental demonstration of the existence of a potential difference was made in a system conforming to the requirements postulated by Donnan. Since Donnan's reasoning was based on the laws of thermodynamics, of course it may be said that the experimental verification of his equation, connecting membrane potentials with ion concentrations, is simply a consequence of the laws of thermodynamics. The same might be said of the experimental verification of Nernst's formula for the E.M.F. of a concentration cell, or of almost any other of the classical experiments of physical chemistry.

Hill's reasoning to the effect that the P.D. between A_H and B_H must be zero is correct, but he seems to forget that the very condition by which this equilibrium is rendered possible is the presence of a membrane impermeable to protein ion. One is not justified in imagining, as Hill does, "that for any physical reason whatever, not *necessarily* because of the existence of a Donnan Equilibrium involving an indiffusible ion, there is a difference of potential between two solutions in equilibrium with one another." In these experiments the difference of potential must depend on the fact that the protein cannot get through the membrane, since that is the only thing that prevents the solutions from being identical at equilibrium. We have the charged or ionic protein, and the membrane impermeable to it; these are the conditions necessary for the existence of a Donnan equilibrium.

The striking facts which Loeb has discovered are the existence of a measurable difference in ion concentrations and of a corresponding measurable difference in potential between the solutions on the two sides of the membrane. These facts can be satisfactorily explained by Donnan's theory, and Loeb has shown how they can be used to explain other properties of protein solutions, notably, the effect of electrolytes on osmotic pressure. Hill has offered no alternative explanation for these facts. Loeb has explained them quantitatively; unless some better quantitative explanation can be proposed, the applicability of Donnan's theory will stand.

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VALENCY RULE AND ALLEGED HOFMEISTER SERIES IN THE COLLOIDAL BEHAVIOR OF PROTEINS.

I. The Action of Acids.

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I.

INTRODUCTION.

It has generally been held in the colloidal literature that different anions of the same valency have different effects on such colloidal properties of proteins as swelling, viscosity, or osmotic pressure, and that the anions could be arranged in definite series according to their influence on these properties. Such series are known under the name of the Hofmeister series. A number of authors have published such series, but the series of no two authors agree. Pauli[1] gives the following series for the influence of acids on the viscosity of blood albumin,

HCl > monochloroacetic > oxalic > dichloroacetic > citric > acetic > sulfuric > trichloroacetic acid

where HCl increased the viscosity most and trichloroacetic least.

Loeb has shown in a series of investigations that the Hofmeister series, as far as they refer to colloidal behavior of proteins, are generally the result of a methodical error; namely, the failure of the authors to measure the hydrogen ion concentration of their protein solutions or protein gels, and the failure to compare the effect of acids on the properties of proteins at the same hydrogen ion concentration of the protein solution or protein gel.[2] When this error is avoided, it

[1] Pauli, W., Kolloidchemie der Eiweisskörper, Dresden and Leipsic, 1920, 60.

[2] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85, 247, 391.

is found that only the valency but not the nature of the anion of an acid has any direct influence on the above mentioned properties of the proteins, and that the Hofmeister ion series have in that case no real existence.

The substitution of the valency rule for the erroneous Hofmeister series gave the clue for the cause of the peculiar influence of electrolytes on these colloidal properties. There is one condition under which only the valency but not the chemical differences of anions can influence the physical properties of protein solutions or protein gels; namely, where membrane equilibria determine these properties.

Donnan[3] had shown that when a solution of two electrolytes is separated from water by a membrane permeable to all but one type of ions, an equilibrium condition results at which the molar concentration of the diffusible crystalloidal ions is not the same on opposite sides of the membrane. Suppose that there is inside a collodion bag a solution of gelatin chloride of a certain pH, then this solution contains in addition to H and Cl ions of the free HCl present in the gelatin chloride solution, positive gelatin ions and the Cl ions combined with the gelatin ions. (There is also non-ionogenic (isoelectric) gelatin, in a certain range of pH but this has no influence on the result.) Only the H and Cl ions can diffuse through the membrane, while the latter is impermeable to the gelatin ions. In such a case, according to Donnan, H and Cl ions should diffuse into the outside solution in such a way that at equilibrium the product of the molar concentrations of the pair of oppositely charged diffusible ions (in this case H and Cl) is the same on the opposite sides of the membrane. Inside the gelatin solution there exists the Cl in combination with the H of the free acid and with the gelatin. If the molar concentration of these Cl ions of the free acid inside the solution is y, the concentration of the H ions is also y. In addition there are the Cl ions in combination with the protein. Let z be the molar concentration of these Cl ions. Hence the total concentration of Cl ions inside the protein solution is $y + z$, while the concentration of H ions is y. The product of the two concentrations is $y\ (y + z)$.

In the outside aqueous solution there is only free HCl, since no gelatin ions can diffuse through the membrane. Let x be the molar

[3] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572.

concentration of the free H ions outside, then the molar concentration of the Cl ions is also x and the product of their concentrations is x^2. According to Donnan, equilibrium is established when

$$x^2 = y\,(y + z) \tag{1}$$

It follows from this equation that the sum of the diffusible ions must be greater inside the solution than outside, but must vary with the hydrogen ion concentration, and this variation causes the influence of electrolytes on the physical properties of proteins. Procter and Wilson[4] have shown that the influence of HCl on the swelling of gelatin can be explained quantitatively from Donnan's equation, and Loeb[5] has shown that the same is true for the influence of acids on osmotic pressure, viscosity, and membrane potentials of protein solutions.

Donnan's equation (1) is a quadratic equation when the anion of the acid is monovalent; Loeb[6] has shown that the equation becomes one of the third degree when instead of HCl a strong dibasic acid such as H_2SO_4 is added. Let x again be the molar concentration of hydrogen ions in the outside solution and y the molar concentration of the hydrogen ions inside the protein solution; then $\frac{x}{2}$ is the concentration of the SO_4 ions in the outside solution and $\frac{y}{2}$ the molar concentration of SO_4 ions of the free H_2SO_4 in the inside (gelatin) solution. The molar concentration of SO_4 ions in combination with gelatin becomes $\frac{z}{2}$. Then the equilibrium equation must be as follows:

$$x^2 \cdot \frac{x}{2} = y^2 \left(\frac{y}{2} + \frac{z}{2}\right)$$

or

$$x^3 = y^2(y + z) \tag{2}$$

If the two equations for membrane equilibria, (1) or (2), are the theoretical basis for the influence of acids on the colloidal behavior of proteins, it is clear that the valency of the anion of an acid must have

[4] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667, 691, 827; 1921–22, iv, 73, 97.

[6] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 351.

a great influence, since it changes the degree of the equilibrium equation; while different ions of the same valency should all have the same influence, since the equation is the same for all anions of the same valency. Exceptions to this conclusion should be found only when the anions cause some secondary chemical or physical change in the constitution of the protein. In other words, if Donnan's theory of membrane equilibria is the true explanation of the influence of acids on the colloidal properties of proteins, it follows that only differences in the valency of the anion of the acid and no other differences in the nature of the anion of acids should, as a rule, influence those four properties of proteins which seem to depend on membrane equilibria, namely membrane potentials, osmotic pressure, swelling, and viscosity, and which constitute the typically colloidal behavior of proteins.

In former experiments Loeb had shown that this was the case. Since, however, the theory of colloidal behavior is thus intrinsically linked with the valency rule, it seemed of importance to make the proof as complete as possible, and this paper intends to offer additional evidence for the valency rule and the non-validity of the Hofmeister series for the colloidal behavior of proteins.

II.

The Necessity of Measuring the pH of the Protein Solution.

If the Donnan equation explains the colloidal behavior of proteins, it is necessary to compare the influence of acids not only for the same concentration of originally isoelectric protein but also for the same pH. The titration curves of isoelectric protein with acid show that when acid is added to isoelectric protein, protein salts are formed which are strongly hydrolyzed.[7] Hence, there exists in a solution of a protein salt an equilibrium between free dissociated acid, protein salt, and non-ionogenic protein. The relative amount of protein salt (or ionized protein) varies with the pH. At the same pH the same proportion of protein is in an ionized condition. The Donnan effect depends on the ionized portion of the protein and the valency of the anion of the acid. As long as the concentration of ionized

[7] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85.

protein in a solution or a gel is the same, and if no salts are present, the solution will have the same osmotic pressure, viscosity, etc., regardless of the nature of the acid, except the valency of its anion (unless the anion causes a secondary chemical or physical alteration of the protein).

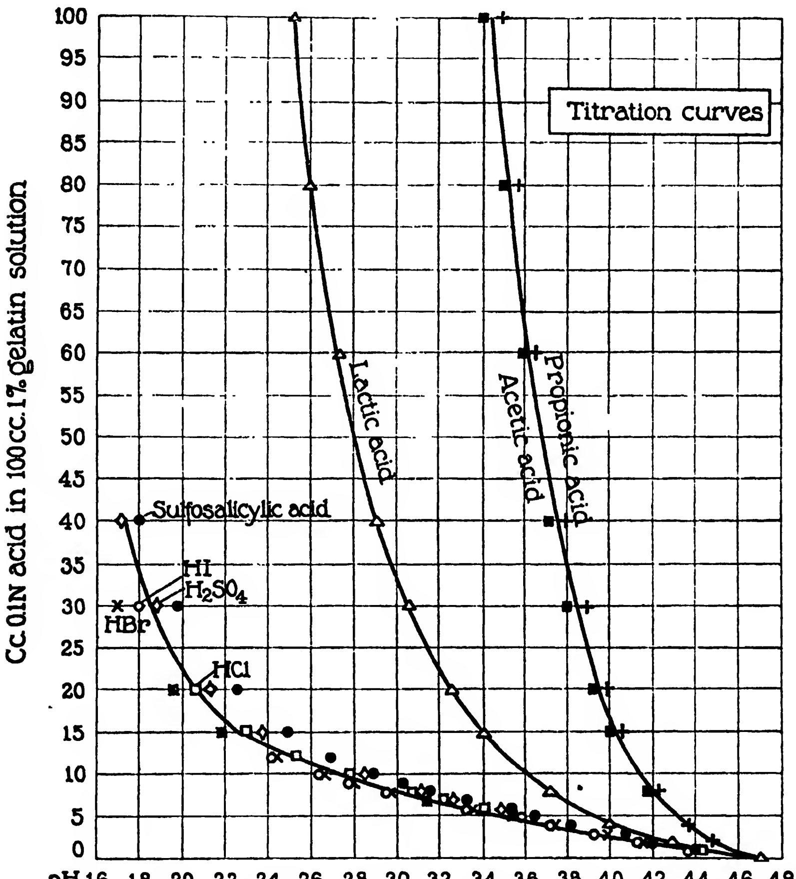

FIG. 1. Titration curves of different acids with solutions containing 1 gm. dry weight of originally isoelectric gelatin in 100 cc. The ordinates are the cc. of 0.1 N acid in 100 cc. of gelatin solution; the abscissæ the pH of gelatin solutions.

The amount of acid required to bring a 1 per cent solution of originally isoelectric protein, *e.g.* gelatin, to the same pH varies with the nature of the acid as is indicated by the titration curves in Fig. 1.

In Fig. 1 are given the titration curves of 1 gm. dry weight of originally isoelectric gelatin in 100 cc. aqueous solution for 0.1 N HCl, HBr, HI, H_2SO_4, and sulfosalicylic acid, and also for the three weak monobasic acids, lactic, propionic, and acetic. As was to be expected, the titration curves for HCl, HBr, and HI are practically identical with those for the two strong dibasic acids, H_2SO_4 and sulfosalicylic; or in other words, the two strong dibasic acids combine in equivalent proportions with the protein. In the case of weak monobasic acids, higher concentrations of acid have to be added to bring the protein solution to the same pH. While 5 cc. 0.1 N of all the strong acids must be contained in 100 cc. solution of originally 1 gm. dry weight isoelectric gelatin to bring the pH to 3.6, 10 cc. of 0.1 N lactic acid are required for this purpose, and 65 cc. of 0.1 N acetic or propionic acid. Yet the concentration of ionized gelatin is the same in all acid solutions at the same pH, regardless of the nature of the acid.

The pH indicates the molar concentration of the free ionized acid which does not combine with the gelatin. By deducting this concentration from the total concentration of the ionized acid added, it is possible to calculate the concentration of a strong acid combined with the protein. All these facts have already been discussed in Loeb's previous papers[7] and book[8] and are here only repeated for the convenience of the reader.

III.

The Influence of Acids on the Membrane Potentials of Gelatin Solutions.

1 gm. dry weight of originally isoelectric gelatin was dissolved in 100 cc. water containing various amounts of 0.1 N acid. Collodion bags of a volume of about 50 cc. were filled with such solutions. The opening of the bag was closed with a rubber stopper perforated by a glass tube serving as a manometer. The collodion bag was put into 350 cc. of an aqueous solution of the same acid and the same pH as that inside the collodion bag, but the outside aqueous solution was free from protein. The temperature was 24°C. After 18 hours or more, *i.e.* after osmotic equilibrium was reached, the membrane potential,

[8] Loeb, J., Proteins and the theory of colloidal behavior, New York and London 1922.

i.e. the P.D. between the protein solution and the outside aqueous solution free from protein, was measured with two saturated KCl calomel electrodes. The E.M.F. of the following cell was therefore measured in these experiments.

Hg	HgCl	saturated KCl solution	gelatin acid solution +	collodion membrane	outside aqueous solution −	saturated KCl solution	HgCl	Hg

The E.M.F. thus measured with identical calomel electrodes we shall call the membrane potential. In Fig. 2 the membrane potentials of

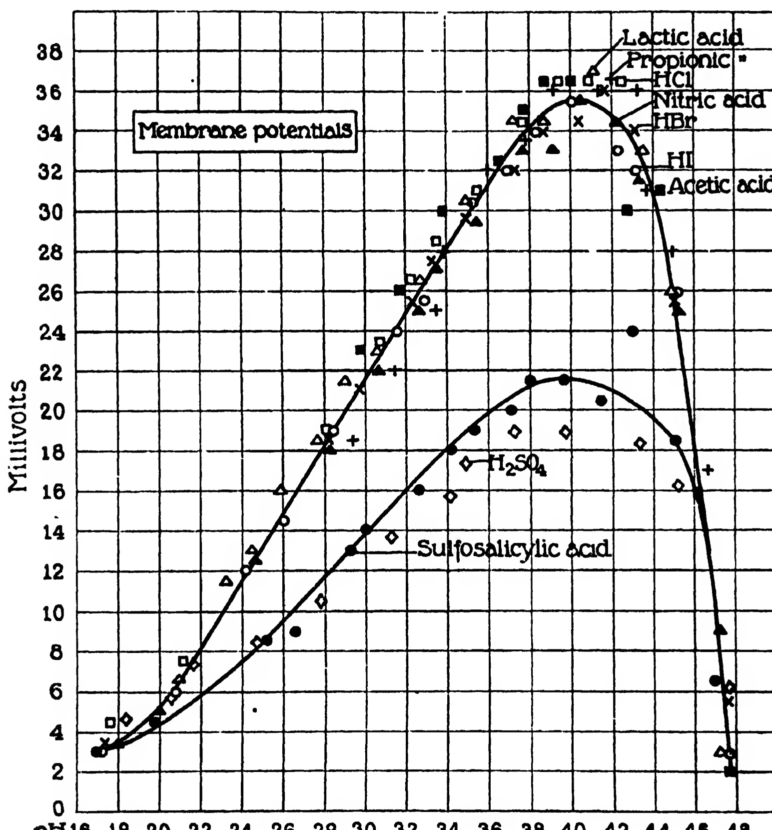

FIG. 2. Proof that only the valency of the anion of an acid influences the membrane potentials of gelatin solutions. The ordinates are the membrane potentials in millivolts; the abscissæ the pH of gelatin solutions. The membrane potentials of the seven monobasic acids are practically identical and so are the membrane potentials of the two strong dibasic acids.

the gelatin solution (*i.e.* the P.D. between gelatin solution and outside aqueous solution across the collodion membrane) are plotted as ordinates over the pH of the gelatin solution at equilibrium. (It should be stated that the pH at equilibrium is, as a rule, different from the pH at the beginning.)

The curves for the membrane potentials in Fig. 2 are as clear an expression of the valency rule and as clear a contradiction of the Hofmeister series as one could expect. It is true that nobody had claimed the validity of the Hofmeister series for the membrane potentials of protein solutions, the reason being that Loeb was the first to measure these potentials for protein solutions, and his experiments showed that the valency rule held. This is confirmed by the results in Fig. 2, since the curves for the membrane potentials of protein solutions are identical in the presence of all monobasic acids used; these acids were in this case HCl, HBr, HI, HNO_3, acetic, propionic, and lactic acid.

The two strong dibasic acids used in these experiments were H_2SO_4 and sulfosalicylic acid. The curves for the membrane potentials of these two strong dibasic acids are also identical, but entirely different from the P.D. curves for the monobasic acids. In other words, *the influence of the acids on the membrane potentials of protein solutions is exclusively a function of the valency but independent of the nature of the anion of the acid.*

IV.

Proof That the Valency Effect of the Membrane Potential Is Determined by Donnan's Equation.

Donnan's equilibrium, equation (1), for monobasic acids can be written in the form

$$\frac{x}{y} = \frac{(y + z)}{x} \tag{1}$$

Donnan has shown that there must exist between the inside and outside solution a P.D. as follows:

$$\text{P.D.} = \frac{R\,T}{F} \log \frac{x}{y}$$

where x is the molar concentration of hydrogen ions outside, and y the molar concentration of hydrogen ions inside. Since pH outside

is $-\log x$ and pH inside $-\log y$, the membrane potential measured with the indifferent calomel electrodes should be equal to the hydrogen electrode potential between the gelatin solution and the outside

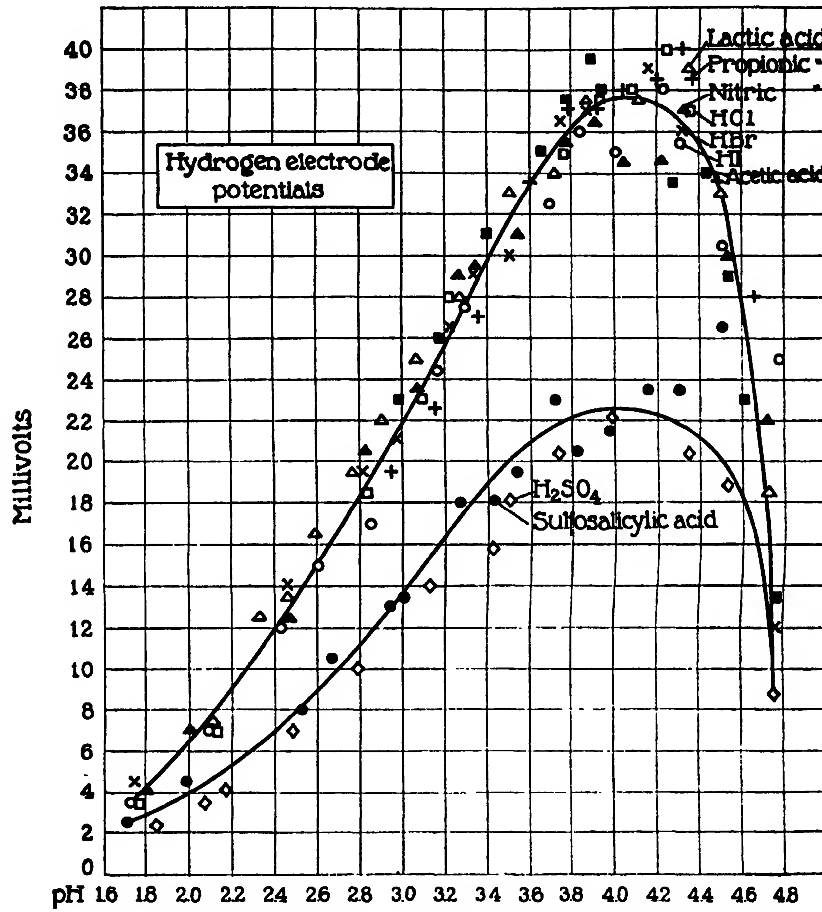

FIG. 3. Proof that the influence of acids on the hydrogen electrode potentials of gelatin solutions is identical with that on the membrane potentials as shown in Fig. 2.

aqueous solution, if the membrane equilibria are the cause of the membrane potentials. This was found to be true within the limits of the accuracy of the measurements (about 2 millivolts).

In Fig. 3 are given the hydrogen electrode potentials of the same acids as in Fig. 2. The curves for the hydrogen electrode P.D. in

Fig. 3 and the curves for the membrane P.D. in Fig. 2 are identical. The hydrogen electrode P.D. for all the monobasic acids are the same within the limits of accuracy of measurements and the P.D. for the two strong dibasic acids are also the same in both Figs. 2 and 3.

Loeb[6] had shown furthermore that if Donnan's membrane equilibrium is responsible for the effect of acids on the membrane potentials of protein solutions, the effects of monobasic acids should be exactly 50 per cent higher than those for dibasic acids at the same pH on the basis of the following consideration.

From equation (1) it follows that in the case of monobasic acids $x = \sqrt{y(y + z)}$. Substituting this value for x in the term $\frac{x}{y}$ we get

$$\frac{\sqrt{y(y+z)}}{y} = \sqrt{\frac{y+z}{y}} = \sqrt{1+\frac{z}{y}}$$

Hence, the membrane potential of a protein solution should be at 24° for monobasic acids

$$\text{P.D.} = \frac{58}{2} \log \left(1 + \frac{z}{y}\right) \text{ millivolts}$$

From equation (2) it follows that in the case of dibasic acids

$$x = \sqrt[3]{y^2(y+z)}$$

Substituting this value in $\frac{x}{y}$ we get

$$\frac{x}{y} = \frac{\sqrt[3]{y^2(y+z)}}{y} = \sqrt[3]{\frac{y^2(y+z)}{y^3}} = \sqrt[3]{\frac{y+z}{y}} = \sqrt[3]{1+\frac{z}{y}}$$

The P.D. is therefore in the case of a dibasic acid

$$\text{P.D.} = \frac{58}{3} \log \left(1 + \frac{z}{y}\right) \text{ millivolts}$$

Hence, at the same pH the P.D. of gelatin sulfate must be to that of gelatin chloride as 2:3, or 0.66.

A comparison of the effects of sulfosalicylic acid with those for HCl and the other monobasic acids at the same pH in Fig. 2 shows that this is correct within the limits of experimental accuracy, except

near the isoelectric point, where the values for the monobasic acids are a trifle too high (see Table I).

TABLE I.

Membrane Potentials for Dibasic and Monobasic Acids.

pH	Dibasic acids.	Monobasic acids.	Ratio $\frac{\text{dibasic}}{\text{monobasic}}$.
	millivolts	*millivolts*	
2.4	7.6	11.4	0.67
2.6	9.6	14.8	0.65
2.8	11.6	18.0	0.64
3.0	13.6	21.6	0.65
3.2	15.8	24.8	0.64
3.4	18.0	28.0	0.62
3.6	19.8	31.0	0.64
3.8	21.2	34.2	0.62
4.0	21.6	35.5	0.61
4.2	20.8	34.8	0.60
4.4	19.2	31.0	0.62

These values are as striking a confirmation of the derivation of the valency rule from the theory of membrane equilibria as could be desired. The values for sulfosalicylic acid were used in preference to the values for sulfuric acid for the reason that a repetition of the experiment with sulfuric acid showed that the values for sulfosalicylic and sulfuric acids are in reality identical, and that the values for sulfuric acid given in Fig. 2 are a little too low.

TABLE II.

Hydrogen Electrode Potentials for Dibasic and Monobasic Acids.

pH	Dibasic acids.	Monobasic acids.	Ratio $\frac{\text{dibasic}}{\text{monobasic}}$.
	millivolts	*millivolts*	
2.6	9.9	15.4	0.64
2.8	11.4	18.6	0.61
3.0	14.0	22.0	0.64
3.2	16.6	25.6	0.65
3.4	19.2	29.4	0.66
3.6	21.0	33.0	0.64
3.8	22.0	36.0	0.61
4.0	22.6	37.5 uncertain.	0.60
4.2		Uncertain.	
4.4	21.4	36.0	0.60

Table II shows that the 2:3 ratio holds also for the hydrogen electrode P.D. values expressed in Fig. 3.

Disregarding the values for the monobasic acids at pH 3.8 to 4.2, where the values for the monobasic acids are a little too high and not quite certain, the ratio of P.D. for the effect of dibasic acid to that of monobasic acid is as nearly 2:3 as the accuracy of the measurements permits. There can therefore, be no doubt that the membrane potentials are determined by the Donnan equilibrium, and, if this be true, the valency rule must hold and there can be no room for any Hofmeister anion series in this case.

The action of weak dibasic and tribasic acids has already been referred to and it only remains to make the statement more complete. Most weak dibasic and tribasic acids dissociate as monobasic acids below a certain pH. H_3PO_4 dissociates as monobasic acid below pH 4.7 and it had been shown that in this range of pH the influence of H_3PO_4 on membrane potentials (as well as osmotic pressure, swelling, and viscosity) is identical with that of HCl or any other monobasic acid if compared for the same pH of the protein solution or gel.[8] Oxalic acid dissociates as a monobasic acid below pH 3.0 and it had been shown that for pH of 3.0 or less the influence of the oxalic acid on the properties mentioned is like that of HCl. Above pH 3.0 the second H ion of the oxalic acid begins to dissociate and the relative number of dibasic anion increases with a further increase of pH and hence the depressing effect of the dibasic anion is felt more and more the higher the pH.[8]

After these remarks, the effect of succinic, citric, and tartaric acids on the membrane potentials as plotted in Fig. 4 is easily understood. All three acids act like HCl below pH 3.0, *i.e.* the curve representing the influence of these three acids on the membrane potential coincides with that for HCl, but not with that for H_2SO_4, which means that all these acids dissociate for pH < 3.0 as monobasic acids, and furthermore, it is clear that these weak dibasic acids behave as the valency rule demands.

Above pH 3.0 the curves for succinic, citric, and tartaric acids are lower than the curve for HCl but considerably higher than that for H_2SO_4, which means that at a pH > 3.0 the second H ion begins to be split off, and the more the stronger the acid. Thus in the case of

the weak succinic acid only a very small percentage of molecules dissociates as dibasic acid and the same may be said for citric acid,

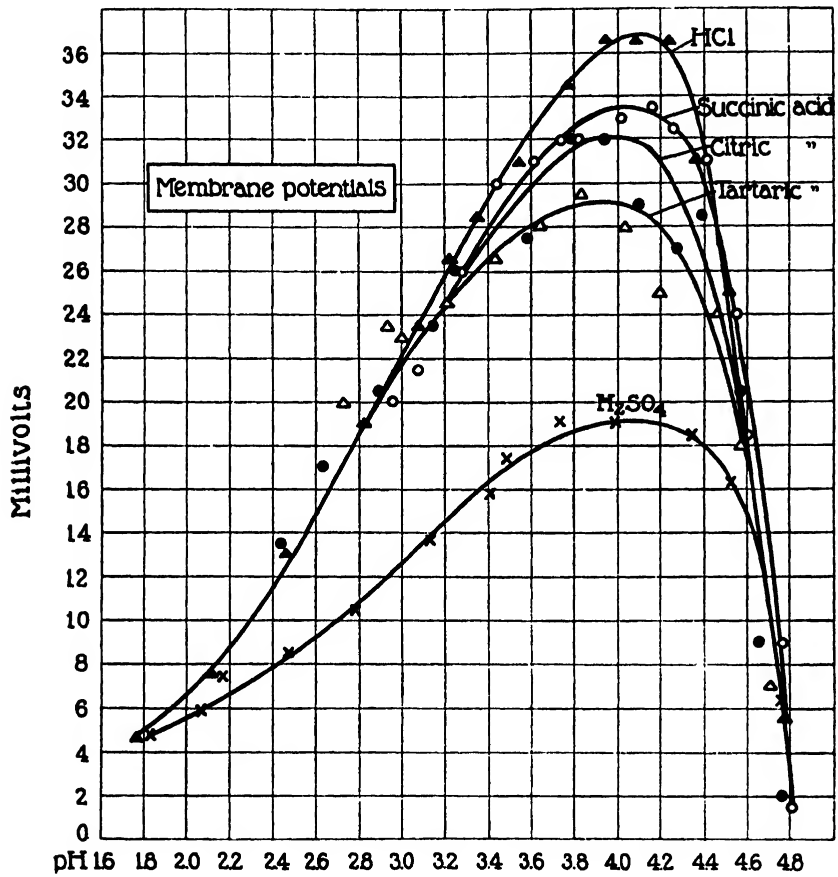

FIG. 4. Influence of weak dibasic and tribasic acids on the membrane potentials of gelatin solutions.

while a greater percentage of tartaric acid molecules dissociates as dibasic acid between pH 3.0 and 4.7. These experiments might almost be used as a criterion for the mode of dissociation of weak dibasic and tribasic acids.

Fig. 5 gives the influence of the same acids on the hydrogen electrode potentials. It is obvious that the membrane potentials observed

with the indifferent calomel electrodes are within the limits of accuracy of measurements and calculations identical with the hydrogen electrode potentials, thus confirming again Donnan's theory of membrane potentials.

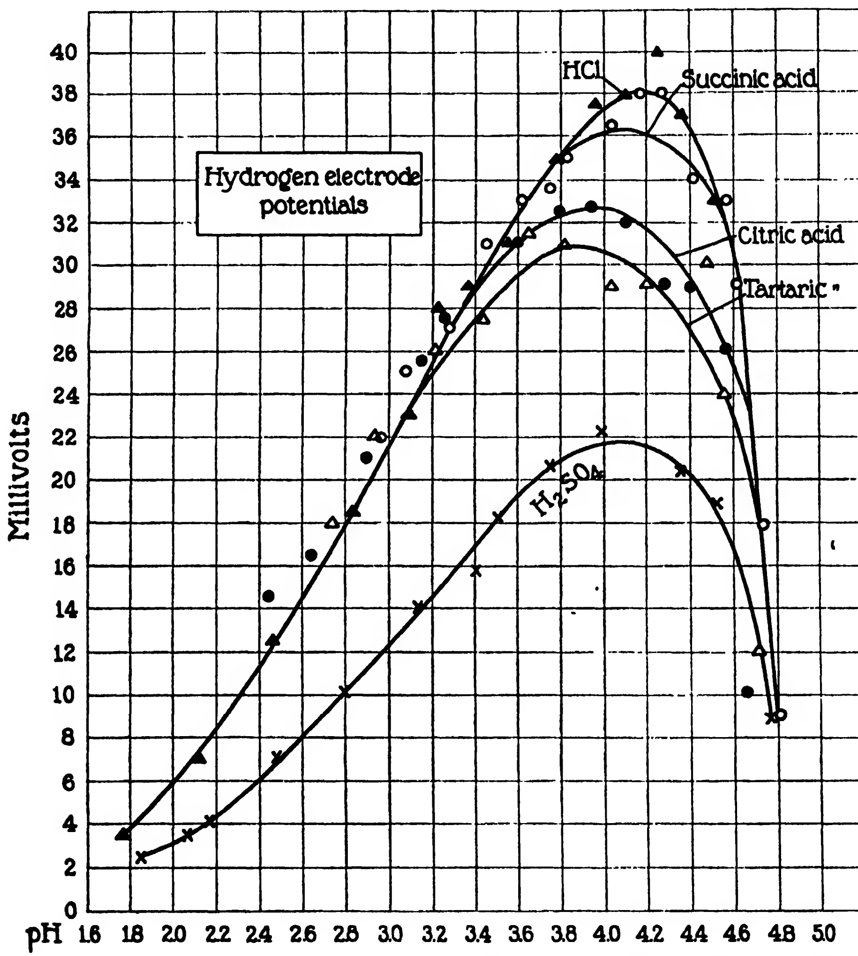

FIG. 5. Influence of weak dibasic and tribasic acids on the hydrogen electrode potentials of gelatin solutions. Notice identity of curves in Figs. 4 and 5.

All these experiments leave no doubt of the experimental and theoretical correctness of the valency rule for membrane potentials and the non-validity of the so called Hofmeister series.

V.

The Valency Effect in the Influence of Acids on the Osmotic Pressure of Protein Solutions.

The same experiments which were used for the measurement of membrane potentials were also used for measuring the osmotic pres-

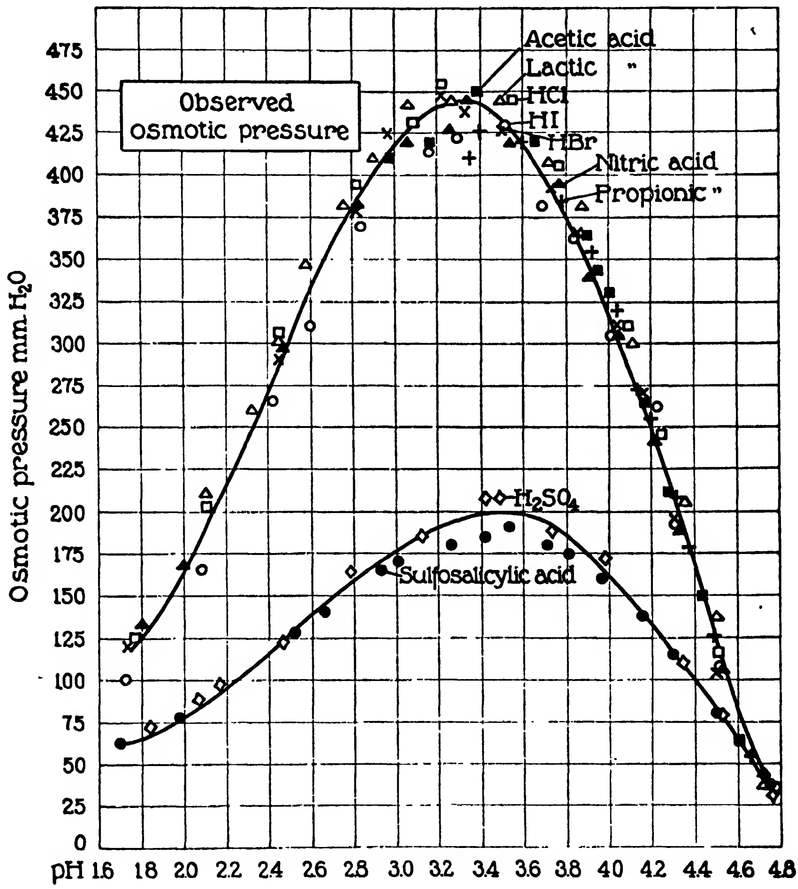

FIG. 6. Proof of valency rule for the influence of acids on the osmotic pressure of gelatin solutions. The influence of seven monobasic acids on the osmotic pressure of gelatin solutions is the same and about twice as high as that of the two dibasic acids.

sure of gelatin solutions containing 1 gm. dry weight of originally isoelectric gelatin in 100 cc. of water made up with various acids. The

results are contained in Fig. 6. The ordinates are the observed osmotic pressures in terms of mm. of a column of water, and the abscissæ are the pH of the gelatin solution at equilibrium. The result is so striking that it leaves no doubt that the valency rule is correct. All the monobasic acids influence the osmotic pressure in exactly the same way; and the values for HCl, HBr, HI, HNO_3, acetic, propionic, or lactic acids lie practically all on one curve. The osmotic pressure curves for the two strong dibasic acids, H_2SO_4 and sulfosalicylic acid, also fall on one curve, which is, however, entirely different, being about half as high as the curve for the monobasic acids for the same pH.

It had been shown in preceding papers and in a book[8] that the curve representing the influence of H_3PO_4 on the osmotic pressure of a gelatin solution is identical with the curve representing the influence of HCl, if both are plotted over the pH of the gelatin solution as abscissæ; and that the curve for oxalic acid is also identical with the curve for HCl and H_3PO_4 for pH 3.0 or below, while for pH above 3.0 the influence of the bivalent oxalate anion becomes noticeable in the fact that the osmotic pressure for oxalic acid is in that range of pH lower than for HCl.

Fig. 7 represents the influence of succinic, citric, and tartaric acids on the osmotic pressure of a solution containing 1 gm. dry weight of originally isoelectric gelatin in 100 cc. solution. As was to be expected, the descending branches of the curves for these acids are identical with the corresponding part of the curve for HCl for pH below 3.0, while above pH 3.0 the curves for the three weak dibasic or tribasic acids are slightly lower in the order of their relative strength as discussed in connection with the membrane potentials.

It would be possible to use the influence of dibasic or tribasic acids on the osmotic pressure of protein solutions to determine their relative strength.

Since it had already been shown previously that the effect of acids on the osmotic pressure of protein solutions is due to the excess of the molar concentration of diffusible ions inside the protein solution over that in the outside solution caused by the Donnan equilibrium, it is mathematically necessary that there should be only an effect of the valency of the anion of the acid on the osmotic pressure but no effect

of the nature of the anion except the valency. Figs. 6 and 7, in addition to the data already published, prove beyond doubt the correctness of this statement.

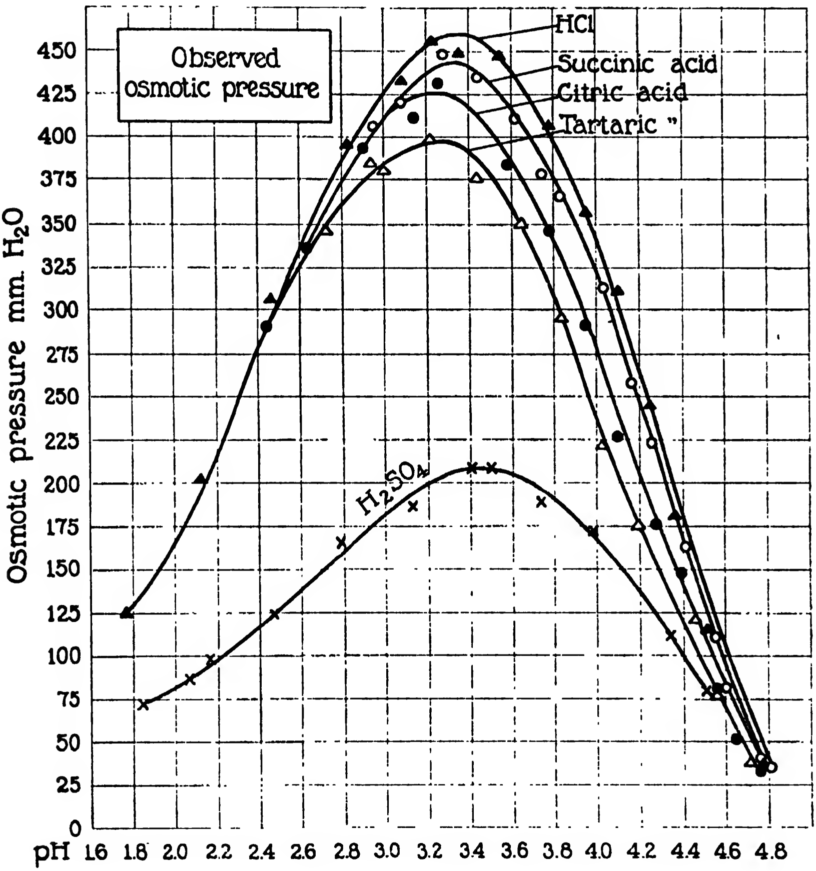

FIG. 7. Influence of weak dibasic and tribasic acids on the osmotic pressure of gelatin solutions.

VI.

Swelling.

Procter and Wilson[4] have shown that the influence of HCl on swelling of gelatin is a purely osmotic effect. The acid combining with the gelatin causes salt formation, the gelatin ions being pre-

vented from diffusing by the cohesive forces between the gelatin ions or molecules of the gel. Since the gel is freely permeable to water and crystalloidal ions, such as H and Cl, the non-diffusibility of the gelatin ions causes the establishment of a Donnan equilibrium between gelatin and outside solution, as a result of which the total molar concentration of all the diffusible crystalloidal ions is greater inside than outside the gel. This causes the influence of the acid on the swelling of gelatin and this influence is the same as that on osmotic pressure for the reason that influence of acid on swelling is also an osmotic pressure effect. The difference between the effect of acid on the osmotic pressure of gelatin solutions and on the swelling of gelatin gels is simply this, that in the former case the diffusion of the gelatin ions is blocked by the collodion membrane and in the latter case by the cohesive forces between the gelatin molecules or gelatin ions of the gel.

These cohesive forces are also the limiting force to the swelling of a gel. Isoelectric gelatin absorbs a certain quantity of water, due to forces which have nothing to do with the Donnan equilibrium, since at the isoelectric point protein is only slightly ionized. The absorption of water by isoelectric gelatin is determined by forces of attraction between certain groups of the gelatin molecule and water, and is primarily though, perhaps, not exclusively a case of solid solution. The additional swelling caused by the addition of acid is, however, as Procter and Wilson have shown, an osmotic phenomenon due to the excess in the concentration of H and Cl ions inside over that outside. This causes the diffusion of water into the gel. The hydrostatic pressure of the water will force the molecules of the gel apart and this will cause an increase in the forces of cohesion which will oppose the further swelling. To give an idea of the difference between the swelling of isoelectric gelatin and that due to the influence of acid, it may be stated that while 1 gm. dry weight of powdered isoelectric gelatin absorbed about 7 gm. of water, the same gelatin when under the influence of an acid with monobasic anion absorbed about 35 gm. of water at pH 3.2 or 3.0 where the swelling is a maximum. The forces of cohesion between the molecules or ions of the gel may be modified by the solute, *e.g.* the anion of the acid, and when this happens, the pure osmotic pressure effect, due to the

Donnan equilibrium, may not be observed. This was noticed in the effect of acids on the swelling of casein, where it was found that swelling occurs in HCl or HNO_3 but not in trichloroacetic acid. These secondary effects of the anion of the acid or of the undissociated acid on the cohesion of the gel are slight and negligible in the case of a gel of gelatin, and for this reason the validity of the valency rule can easily be demonstrated for the influence of acids on the swelling of gelatin.

The most accurate method for determining the degree of swelling of gelatin is by weighing the gelatin at the beginning and end of the experiment. The earlier experimenters used large blocks of gelatin for the weighing experiment, but in that case it requires days before the maximum of swelling is reached and in the meantime some of the gelatin may have been dissolved. The solution effect of different acids on gelatin is probably not the same and in solubility the nature of the anion may play a considerable rôle. It is therefore obvious that experiments on the swelling of blocks of gelatin cannot well be used for theoretical conclusions. We used powdered particles of gelatin of a definite size of grain; namely, particles which went through a sieve with mesh of $\frac{1}{30}$ but not through mesh $\frac{1}{60}$ of an inch. It was found that such particles reach the maximal swelling in 2 hours. By making the experiment at 15°C. the loss by solution of the gelatin was less than 5 per cent. After the acid had acted for 2 hours at 15°C. on the gelatin, the latter was put on a filter, the solution was allowed to drain off, and the weight of the gelatin was determined. In this way better results could be obtained than with the older method of using a solid block or by estimating the swelling from the volume of the powdered gelatin.

A large quantity of powdered gelatin of the size of grain as stated was brought to the isoelectric point in the way described by Loeb.[8] 8 gm. of the wet isoelectric powdered material contained 1 gm. dry weight of isoelectric gelatin. This mass of powdered isoelectric gelatin in equilibrium with water served as stock material and was kept moist in an ice chest at about three degrees centigrade. Equal portions of 8 gm. each of the stock gelatin, each containing about 1 gm. dry weight of isoelectric gelatin, were put into beakers and allowed to stand for about 18 hours in a moist chamber of 15–16°C. The

8 gm. portions of gelatin were then added each to 150 cc. of H_2O containing various amounts of 0.1 N acids of 15°C. and allowed to stand, with frequent stirring, for 2 hours at 15°C. The gelatin was then removed from the outside solution by filtration through cotton-wool in weighed funnels. The liquid was allowed to drain off completely and the weight of gelatin was determined. The gelatin was afterwards melted at 50°C., cooled to 25°, and its pH was measured electrometrically. All operations, except pH measurements, were done in a constant temperature room at about 15°C. A control consisting of 9 cc. of 0.1 N HCl per 150 cc. of H_2O was used with each

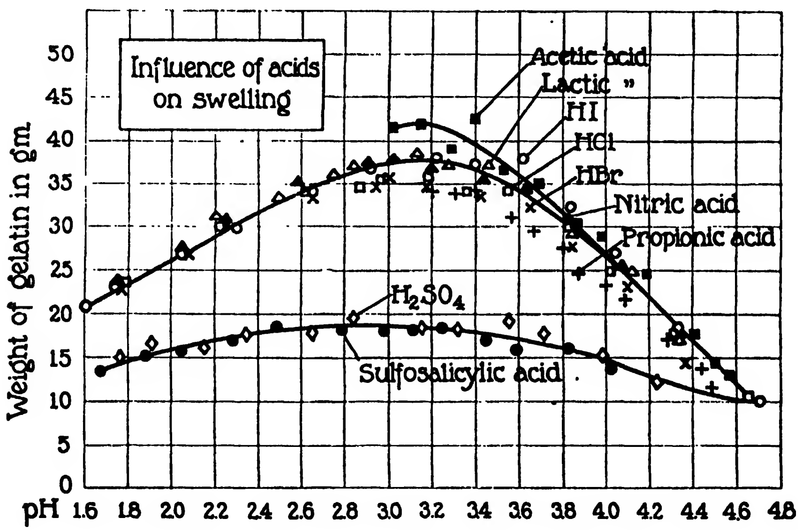

FIG. 8. Proof of valency rule for the influence of acids on the swelling of gels of gelatin. The influence of the seven monobasic acids is (aside from slight secondary effects of acids presumably on the cohesion of the gel) the same and considerably higher than that of the two dibasic acids.

series. The pH of the gelatin of the control was 3.19 and the weight of the control varied mainly between 34 and 36 gm., the extreme variations being 32.2 and 37.8 gm.

Fig. 8 gives the results with different acids. The abscissæ are the pH of the gel at the end of the experiment, while the ordinates are the weight of the gelatin at the end of the experiment. All the values for the influence of the six monobasic acids, HCl, HBr, HI, HNO_3,

propionic, and lactic acid on swelling lie on the same curve within the limits of the accuracy of the experiments, with a maximal weight of about 36 gm., which is inside the variations for the controls with HCl referred to. Only acetic acid gives a slightly higher maximal value of about 42 gm. at pH 3.2. This had been observed by Loeb before. The abnormal behavior of acetic acid does not occur in either membrane potentials or osmotic pressure, where the effects are due to isolated gelatin ions. The suspicion is therefore justified that the excessive effect of acetic acid on swelling is due to a diminution of the cohesion of the gel caused by the high concentration of acetic acid required to bring the pH to 3.2 or 3.0. This idea had already been suggested by Loeb.[8]

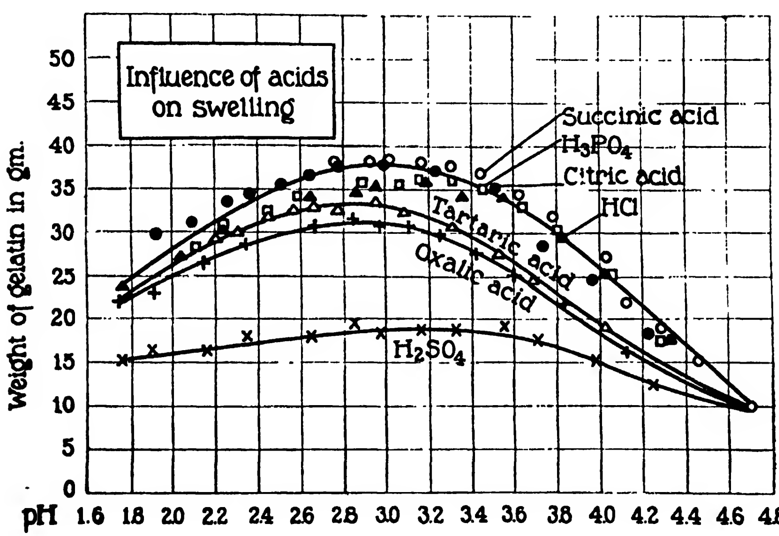

FIG. 9. Influence of weak dibasic and tribasic acids on swelling.

On the other hand, the strong dibasic acids, H_2SO_4 and sulfosalicylic acid, also act alike but cause a maximal weight of only 18 gm., which is about one-half of the maximal weight of the gelatin under the influence of HCl. This ratio of 1:2 for dibasic and monobasic acids is about the same as that observed for the valency effect of anions in the case of osmotic pressure. The maximum lies at a pH of about 3.0 to 3.2 of the gel.

These experiments show that only the valency but not the chemical nature of the anion of the acid influences the swelling of gelatin.

Fig. 9 shows the effect of weak dibasic and tribasic acids on swelling. From what has been said concerning the electrolytic dissociation of these acids it is obvious that their effect on swelling is also as clearly a confirmation of the valency rule as is their action on membrane potentials and on osmotic pressure.

VII.

Viscosity.

Loeb has shown that in the case of colloids we must discriminate between two sources of viscosity;[5] first, the ordinary viscosity of true solutions, and second, the viscosity of solutions containing in addition to molecules and ions solid aggregates capable of swelling. Both forms of viscosity may be influenced by the addition of electrolytes, but the mechanism of the influence is entirely different in the two cases. In the case of true solutions, the addition of electrolytes may influence the attraction between the molecules of solute and the molecules of water. This may explain the observation of Reyher that the viscosity of the solution of salts of fatty acid is greater than the viscosity of the solution of fatty acids, since the former are more ionized. This effect is of a low order of magnitude. When, however, submicroscopic particles of solid jelly are present in a solution of a protein, *e.g.* gelatin or casein, the addition of acid causes a swelling of the particles and this increase in the ratio of relative volume of solute to that of solvent causes an increase of a much higher order in the viscosity than the increase observed in true solutions. Moreover, in this second form of viscosity due to swelling we are dealing with a phenomenon which depends on the Donnan equilibrium. Hence in this case the valency rule should apply, while in the influence of acids on the viscosity of solutions containing no solid particles, the chemical nature of the anion of an acid might be expected to be of great influence. Since a solution of gelatin contains isolated molecules of gelatin as well as suspended particles, both forms of viscosity will be influenced by acids. But the influence of acid on the swelling of the submicroscopic particles of jelly causes a much greater increase of viscosity than the

influence of acid on the isolated molecules so that we may neglect the latter effect.

The method of procedure was as follows. A stock solution of 2 per cent isoelectric gelatin was made up and kept in the ice chest at about 3°C. A portion of the stock of the 2 per cent isoelectric gelatin was liquefied by heating to about 45°C. and then kept warm (at about 35°C.) during each experiment in order to avoid solidification. 25 cc. of the 2 per cent isoelectric gelatin were pipetted into a 50 cc. graduated flask, the required amount of acid added from

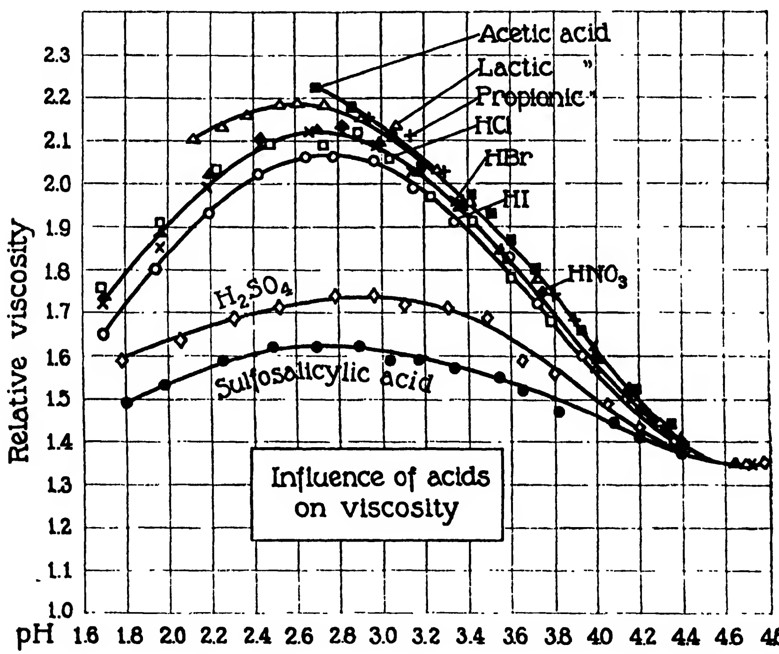

FIG. 10. Proof of the valency rule for the viscosity of gelatin solutions for seven monobasic and two dibasic acids.

a burette, and the volume was then made up to 50 cc. with H_2O. The mixture was heated in a hot water bath to 45°C., rapidly cooled in ice water to 24°C., and the viscosity was immediately measured at 24°C. by means of an Ostwald viscometer.

Fig. 10 gives the influence of the seven monobasic acids, HCl, HBr, HI, HNO_3, lactic, acetic, and propionic acids, and of two strong dibasic

acids, on the relative viscosity of gelatin solutions as described. The validity of the valency rule is obvious. The seven monobasic acids act almost alike, differing entirely from the action of the two strong dibasic acids, H_2SO_4 and sulfosalicylic. The curves are similar to the curves of swelling in Fig. 8 and of osmotic pressure in Fig. 6. This finds its explanation in the fact that this form of viscosity is due to the swelling of particles and that the influence of acids on swelling is an osmotic phenomenon, as demonstrated by Procter and Wilson.

There are, however, some slight discrepancies between the curves for swelling (Fig. 8) and for viscosity (Fig. 10). Thus in Fig. 10 the influence of HI is less than that of HCl, that of sulfosalicylic acid less than that of H_2SO_4. These slight differences are probably due to a secondary influence of the acids possibly on solubility. Gelatin is *e.g.* more soluble in solutions of NaI than of NaCl. In the viscosity experiments the gelatin is heated to 45°C. before the viscosity measurements. In this heating the majority of submicroscopic particles of gel in the solution are completely dissolved but this solution of particles is possibly more complete in HI than in HCl and more complete in sulfosalicylic acid than in H_2SO_4. These secondary effects would explain the appearance of slight differences in the action of acids on viscosity which are lacking in the case of membrane potentials, osmotic pressure, and swelling, where the gelatin is kept at lower temperatures.

The effect of acetic acid on viscosity (Fig. 10) is slightly higher than that of HCl. This was to be expected if the effect of acid on the viscosity of gelatin solution is due to the swelling of submicroscopic particles of gelatin contained in the solution. The same anomaly was also observed in the effect of acetic acid on swelling.

Fig. 11 gives a comparison of the effect of succinic, citric, and tartaric acids with that of HCl on the viscosity of gelatin solutions. The effects differ but slightly for the four acids.

Summarizing the results of the previously published experiments of Loeb and the results of the new experiments, we may say that they show that only the valency of the anion of the acid and none of the other properties of the anion influence the membrane potentials, osmotic pressure, swelling, and viscosity of gelatin; and that, moreover, this result was to be expected theoretically since the influence of acids on the four properties of gelatin is determined by the Donnan equi-

librium which varies with the valency but not with the other properties of the anions of acids.

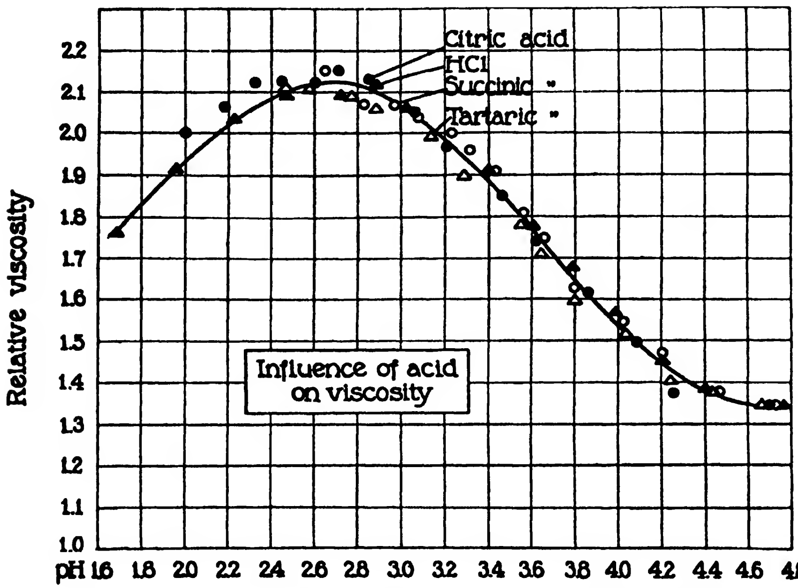

FIG. 11. Influence of weak dibasic and tribasic acids on the viscosity of gelatin solutions.

VIII.

Critical Remarks.

The conclusions reached in the older papers on the influence of acids on swelling, osmotic pressure, and viscosity are due to the fact that the effects of different acids were not compared at the same pH of the protein solution or the gel after equilibrium was reached, but, as a rule, for identical quantities of acid added to the water into which the gel was put. The effects of the same acid were therefore not compared at the same concentration of ionized gelatin in the protein solution or gel (or for the same pH of the gel) but at entirely different pH of the solution or gel. This led to error, so that the nature of the anion of an acid aside from its valency seemed to have an effect, which was in reality the effect of differences in the pH of the gel. Kuhn[9] in a paper published as late as 1921 calculates the H^+ ion concentration of protein

[9] Kuhn, A., *Kolloidchem. Beihefte*, 1921–22, xiv, 147.

gels from Kohlrausch's tables as if the pH in the presence of gelatin were the same as that of acid in water free from gelatin, omitting the consideration of the fact, proved by the titration curves, that part of the acid combines with the gelatin and that this alters the hydrogen ion concentration. He furthermore overlooks the fact that on account of the Donnan equilibrium the pH of the gel is different from that of the surrounding aqueous solution. It is, therefore, necessary to measure the pH of the gel and it is wrong to state that the influence of different acids on swelling has been compared at the same pH when no mention of the Donnan equilibrium has been made and when the pH of the gel itself has not been measured. These and similar errors have led certain authors to imagine that they have proved the reality of the Hofmeister anion series, while they neglected to measure properly the pH of the gel or of the protein solutions.

And finally, Kuhn did not take the trouble to use as standard material isoelectric protein, since this would have required pH measurements. Pauli[1] arrived at his Hofmeister series for the action of acids on the viscosity of solutions of blood albumin by the same mistake as Kuhn, plotting the viscosity over the molar concentrations of acid which the solutions would have had if no protein had been present. Lillie's[10] Hofmeister series for osmotic pressure effects was based on the same error, and the same is true for all the earlier experiments on the effect of acids on the physical properties of proteins.

IX.

SUMMARY.

1. The action of a number of acids on four properties of gelatin (membrane potentials, osmotic pressure, swelling, and viscosity) was studied. The acids used can be divided into three groups; first, monobasic acids (HCl, HBr, HI, HNO_3, acetic, propionic, and lactic acids); second, strong dibasic acids (H_2SO_4 and sulfosalicylic acid) which dissociate as dibasic acids in the range of pH between 4.7 and 2.5; and third, weak dibasic and tribasic acids (succinic, tartaric, citric) which dissociate as monobasic acids at pH 3.0 or below and dissociate increasingly as dibasic acids, according to their strength, with pH increasing above 3.0.

[10] Lillie, R. S., *Am. J. Physiol.*, 1907–08, xx, 127.

2. If the influence of these acids on the four above mentioned properties of gelatin is plotted as ordinates over the pH of the gelatin solution or gelatin gel as abscissæ, it is found that all the acids have the same effect where the anion is monovalent; this is true for the seven monobasic acids at all pH and for the weak dibasic and tribasic acids at pH below 3.0. The two strong dibasic acids (the anion of which is divalent in the whole range of pH of these experiments) have a much smaller effect than the acids with monovalent anion. The weak dibasic and tribasic acids act, at pH above 3.0, like acids the anion of which is chiefly monovalent but which contain also divalent anions increasing with pH and with the strength of the acid.

3. These experiments prove that only the valency but not the other properties of the anion of an acid influences the four properties of gelatin mentioned, thus absolutely contradicting the Hofmeister anion series in this case which were due to the failure of the earlier experimenters to measure properly the pH of their protein solutions or gels and to compare the effects of acids at the same pH of the protein solution or protein gel after equilibrium was established.

4. It is shown that the validity of the valency rule and the nonvalidity of the Hofmeister anion series for the four properties of proteins mentioned are consequences of the fact that the influence of acids on the membrane potentials, osmotic pressure, swelling, and viscosity of gelatin is due to the Donnan equilibrium between protein solutions or gels and the surrounding aqueous solution. This equilibrium depends only on the valency but not on any other property of the anion of an acid.

5. That the valency rule is determined by the Donnan equilibrium is strikingly illustrated by the ratio of the membrane potentials for divalent and monovalent anions of acids. Loeb has shown that the Donnan equilibrium demands that this ratio should be 0.66 and the actual measurements agree with this postulate of the theory within the limits of accuracy of the measurements.

6. The valency rule can be expected to hold for only such properties of proteins as depend upon the Donnan equilibrium. Properties of proteins not depending on the Donnan equilibrium may be affected not only by the valency but also by the chemical nature of the anion of an acid.

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VALENCY RULE AND ALLEGED HOFMEISTER SERIES IN THE COLLOIDAL BEHAVIOR OF PROTEINS.

II. THE INFLUENCE OF SALTS.

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(Received for publication, March 20, 1923.)

Difference between the Action of Acids and of Salts on Colloidal Behavior.

In order to understand the action of salts on colloidal behavior, it is necessary to understand the difference between the action of acids and of salts. When acid is added to a solution of isoelectric protein, the membrane potentials, osmotic pressure, and, in the case of some proteins (*e.g.* gelatin), viscosity, are first increased until a maximum is reached; and when more acid is added, the values representing these properties drop again. The action of acid on the swelling of gelatin is the same, since the swelling due to acid is merely an osmotic phenomenon. This rise and fall of the curves for these four properties finds its explanation through the following facts. The addition of acid to isoelectric protein has two opposite effects; first, a salt formation between protein and acid, which increases the concentration of ionized protein, and this causes a rise in the value of the four properties mentioned with the addition of acid; and second, the depressing influence of the anion of the acid on the value of the four properties. Both effects increase with the increase in the concentration of acid, but not at the same rate. When little acid is added to isoelectric protein, the salt formation and ionization of the protein increases at first more rapidly than the depressing effect of the anion of the acid until a point is reached where the further addition of acid increases the ionization little, and finally not at all, while the depressing effect of the anion of the acid continues to increase. This peculiar action of

the acid finds its explanation in the theory of membrane equilibria, as shown in preceding papers and a book.[1]

If this theory of membrane equilibria is correct, we should expect that, when the protein salt is a salt of the type of gelatin chloride, where the gelatin ion is positively charged, only the anion of an acid (or a salt) should influence the four properties dependent on the membrane equilibrium, namely membrane potentials, osmotic pressure, swelling, and that type of viscosity which is due to the swelling of protein particles; and furthermore, that only the valency of the anion but not its chemical nature should have an effect (unless these properties are affected in an indirect way by a change in the chemical or physical constitution of the protein by a specific acid). The validity of the valency rule has already been discussed for acids and will be discussed more fully here for salts.

The difference between the action of salts and acids on the four properties of protein is this, that while acids (and alkalies) cause ionization of isoelectric protein, salts have no such effect. When a salt is added to a solution of a protein salt, *e.g.* gelatin chloride, of a given pH, only the depressing action of the anion of the salt on these properties is noticed, but no augmenting effect of the cation of the salt. This was proved by Loeb by the following two sets of experiments. When all the protein is ionized, *e.g.* when gelatin chloride of pH 2.5 is chosen, HCl and NaCl act quantitatively alike, since now no further ionization can be produced by the acid. When, however, the pH of the gelatin chloride is higher (but below pH 4.7), the addition of little acid will cause an increase in the values of the four properties which will not be produced by the salt.[1]

The second proof consisted in the fact that if the pH of the protein solution is kept constant, salts of the type of NaCl, $CaCl_2$, and $LaCl_3$ will have an equal depressing effect on the properties of protein-acid salts for the same concentration of the Cl ions of the three salts, at the same pH of the protein solution or protein gel. This was proven for membrane potentials and osmotic pressure.[2]

[1] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

[2] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 752. Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 597.

If the explanation of these effects of salts on the basis of the Donnan equation is correct, it follows furthermore that only the anion of a salt should have a depressing effect on the four properties of protein-acid salts, and only the cation of a salt should have a depressing effect on the properties of metal proteinates. Loeb has already shown that this is correct[1] and it is intended to add in this paper some further experimental evidence. This was considered advisable on account of the fact that some authors continue to maintain that for the action of salts on proteins the Hofmeister series are valid. Those who believe in the validity of the Hofmeister series for the action of salts on the four above mentioned properties of proteins arrange the effect of salts on the swelling of gelatin in the following way

$$SO_4, \text{ tartrate, citrate} < \text{acetate} < Cl < Br, NO_3 < I < CNS$$

where the swelling is said to be a maximum in CNS and a minimum in SO_4. "Above a certain concentration the sulfates, tartrates, and citrates cause a shrinkage of the gel of gelatin, and acetate acts in the same sense but less strongly, while the other anions cause an increasing swelling of the gel of the same concentration."[3] Loeb had shown that these and similar statements were simply due to the failure of the older authors to measure the pH of the gel and that if this quantity is properly measured with the hydrogen electrode, it is found that there exists only an effect of the valency of the anion of the salt on swelling and no effect of the chemical nature of the anion (provided the pH of the protein solution or gel is on the acid side of the isoelectric point).[1]

As far as the actions of the cations of a salt are concerned, the following statement is made. "The differences in the effects of cations are less marked; it might be possible to propose the series $Li < Na < K, NH_4$; then follow the alkali earths with Mg in a position between."

It is also stated that these anion and cation series are correct at a neutral reaction. Since the isoelectric point of gelatin is at pH 4.7, at neutral reaction, *i.e.* pH near 7.0, gelatin exists as metal gelatinate, the properties of which are not affected at all by the anions of a salt

[3] Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 5th edition, 1922, pt. 1, 267.

and are affected strongly by the cation of a salt. This alone shows that the anion series at pH 7.0 must be the result of a methodical error. As far as osmotic pressure is concerned, Lillie[4] states that salts depress the osmotic pressure of neutral solutions of egg albumin in the following order

$$SO_4 > Cl > Br > I > NO_3 > CNS$$

At neutral reaction of the albumin solution the anions of a salt can have no influence on the osmotic pressure of a protein solution. Neither Lillie nor any of the other authors had measured the pH of their solutions or gels, and the Hofmeister series on osmotic pressure and swelling are the result of this methodical error, as a consequence of which these authors mistook effects due to variation of the pH for effects due to the nature of the anion. If we wish to study the effect of anions on the four physical properties of proteins—membrane potentials, osmotic pressure, swelling, and viscosity—we must use protein-acid salts, the pH of which is in the case of gelatin less than 4.7, and we must make sure that the pH of the protein solution or protein gel is not altered by the addition of salt. This can be done only by controlling the pH with the hydrogen electrode, as will be shown in the following experiments.

Membrane Potentials.

For these experiments three stock solutions all of pH 3.8 were prepared: first, solutions of gelatin chloride of pH 3.8 containing 2 gm. dry weight of originally isoelectric gelatin and 8 cc. of 0.1 N HCl in 100 cc. solution; second, M/2 solutions of different salts brought to a pH of 3.8 by the addition of HCl; and third, distilled water brought also to the pH of 3.8 by the addition of HCl. By successive dilution of the M/2 salt solutions of pH 3.8 with distilled water of pH 3.8, series of salt solutions of different degree of concentration, but all of pH 3.8, were prepared. 50 cc. of the 2 per cent solutions of gelatin chloride of pH 3.8 and 50 cc. of the salt solutions of pH 3.8 were then mixed and 1 per cent solutions of gelatin chloride in salt solutions of different concentrations, but all of pH 3.8, were obtained.

[4] Lillie, R. S., *Am. J. Physiol.*, 1907–08, xx, 127.

Collodion bags of about 50 cc. volume were filled with such solutions (closed with stoppers perforated by manometer tubes as described), and the collodion bags were submerged in beakers containing each 350 cc. of the same salt solution as that inside the collodion bags and all of pH 3.8, but without protein. The experiments lasted for 18 to 24 hours at 24°C. At that time osmotic equilibrium was reached, the osmotic pressure was read, and the membrane potentials between the protein solution inside the collodion bag and the outside aqueous solution free from protein were measured with a pair of indifferent calomel electrodes (in saturated KCl) as described.

Fig. 1 represents the effect of six different salts with monovalent anions, NaCl, NaBr, NaI, $NaNO_3$, NaCNS, and Na acetate, and one salt with divalent anion, Na_2SO_4. These salts were selected to find out whether the above mentioned Hofmeister series are real or whether the valency rule holds. The abscissæ are the concentrations of the salts and the ordinates are the observed membrane potentials in millivolts. When no salt was added, *i.e.* when the concentration was zero, the values vary within about 3 to 4 millivolts due to the limits of experimental accuracy. This variation was, of course, also found when salts were added. The curves in Fig. 1 show that the variations between the effects of the six different salts with monovalent anion are chance variations such as are observed when no salt is added, *i.e.* at concentration 0 in Fig. 1, and that if this is taken into consideration it is found that the effects of the six salts on membrane potentials lie all on one curve. The curve for Na_2SO_4 is, however, considerably lower than the curve for the six salts with monovalent anions; namely, a little less than two-thirds. The curves in Fig. 1, therefore, show that the six salts, NaCl, NaBr, NaI, $NaNO_3$, NaCNS, and Na acetate, have (within the limits of accuracy of measurements) the same effect on the membrane potentials, while the depressing effect of SO_4 is about two-thirds or still less. These experiments prove the valency rule but contradict the Hofmeister ion series.

In order to bring 100 cc. of M/2 stock solutions of the different salts to a pH of 3.8, the solutions had to contain different amounts of 0.1 N HCl, as Table I shows.

Attention is called to the enormous amount of HCl required to maintain the pH of 3.8 in the presence of M/2 Na acetate. Yet the

older colloid chemists compared the effect of Na acetate with that of NaCl without correcting for the difference of pH.

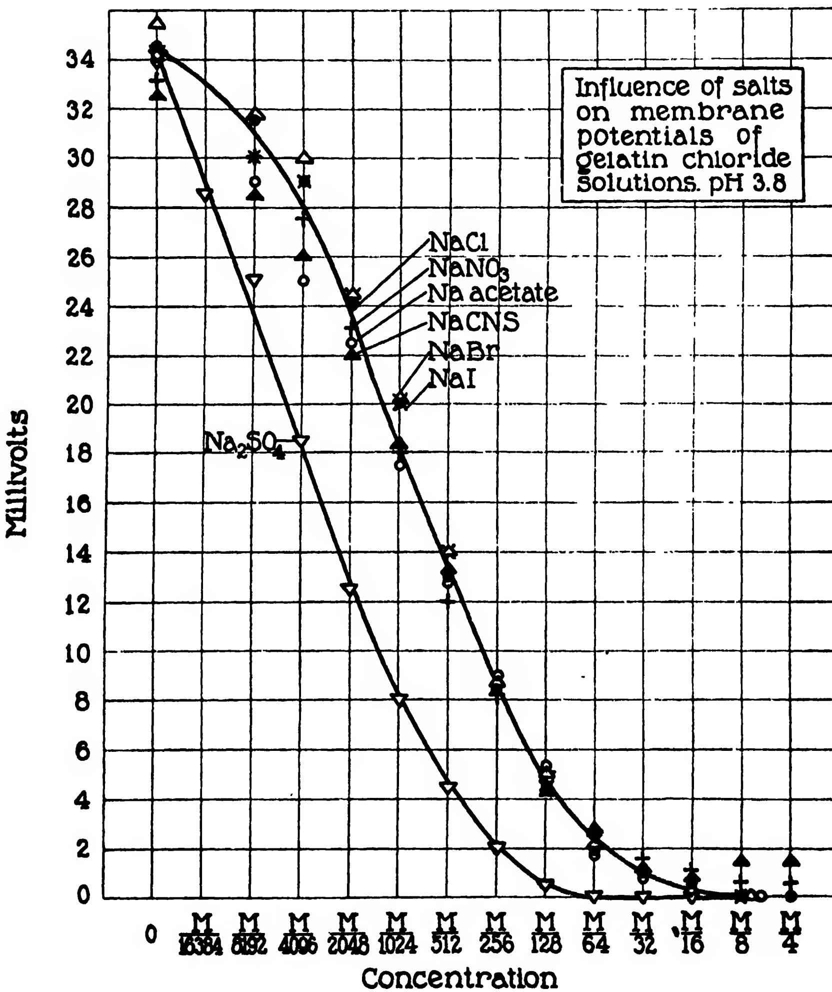

FIG. 1. All salts with monovalent anions have within the limits of experimental accuracy the same depressing effect on the membrane potentials of gelatin chloride solutions at pH 3.8, while the depressing effect of Na_2SO_4 is much greater.

This fact has to be kept in mind when the effect of buffer salts with divalent anions is examined. The following number of cc. of

0.1 N HCl (or their equivalent) was required in 100 cc. of M/2 solutions of such salts to produce a pH of 3.8.

Na_2 oxalate	55.0 cc. 0.1 N HCl
Na_2 tartrate	150.0 cc. 0.1 N HCl
Na_2 succinate	430.0 cc. 0.1 N HCl

Now in these solutions, NaCl and weak dibasic acids are formed, the latter dissociating chiefly, though not exclusively, as monobasic acids. By adding such buffer salts to a solution of gelatin chloride of pH 3.8 and maintaining the pH by adding HCl, part of the bivalent anions of the salts are transformed into monovalent anions. Hence

TABLE I.

Cc. of 0.1 N HCl in 100 cc. M/2 Solutions of Salt Required to Produce a pH of 3.8.

	0.1 N HCl required.
	cc.
NaCl	0.2
NaBr	0.6
NaI	0.3
$NaNO_3$	0.3
NaCNS	0.7
Na acetate	425.0
Na_2SO_4	1.0

the depressing effect of the three above mentioned salts should lie between that of NaCl and that of Na_2SO_4, but that of Na_2 oxalate or Na_2 tartrate nearer to Na_2SO_4 than that of Na_2 succinate. Fig. 2 shows that this is correct.

The older authors ignored the pH and compared the effect of *e.g.* Na_2 tartrate with that of NaCl, disregarding the change in pH. Their error was not one of colloid chemistry, but one of physical chemistry. When the pH of the gelatin solution is kept constant, the influence of salts on membrane potentials of a gelatin chloride solution is determined only by the valency of the anion of the salt but not by its chemical nature.

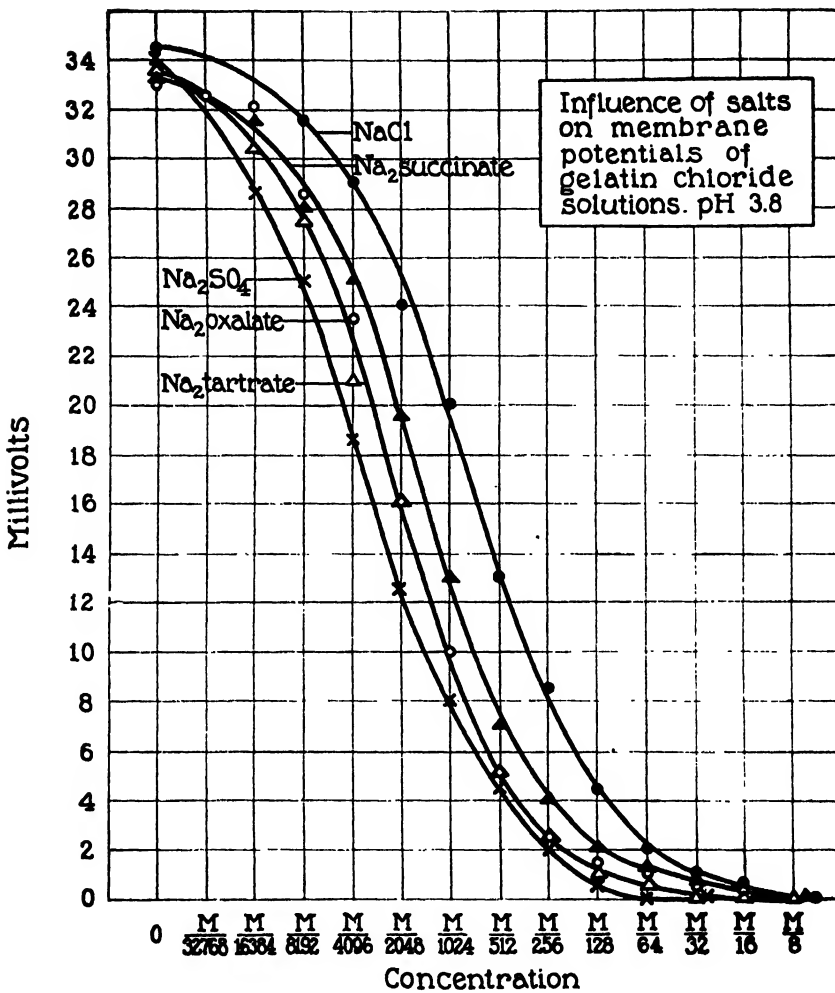

FIG. 2. Effect of strong and weak dibasic salts on the membrane potentials of gelatin chloride solutions at pH 3.8.

Osmotic Pressure.

In the preceding experiments on membrane potentials the influence of the salts on osmotic pressure of the protein solutions at pH 3.8 was also measured. Fig. 3 gives the result. The ordinates are the observed osmotic pressures in terms of a column of water. The de-

pressing effects of the six salts with monovalent anion (NaCl, NaBr, NaI, $NaNO_3$, NaCNS, Na acetate) on the osmotic pressure of the gelatin chloride solutions of pH 3.8 (containing 1 gm. dry weight of

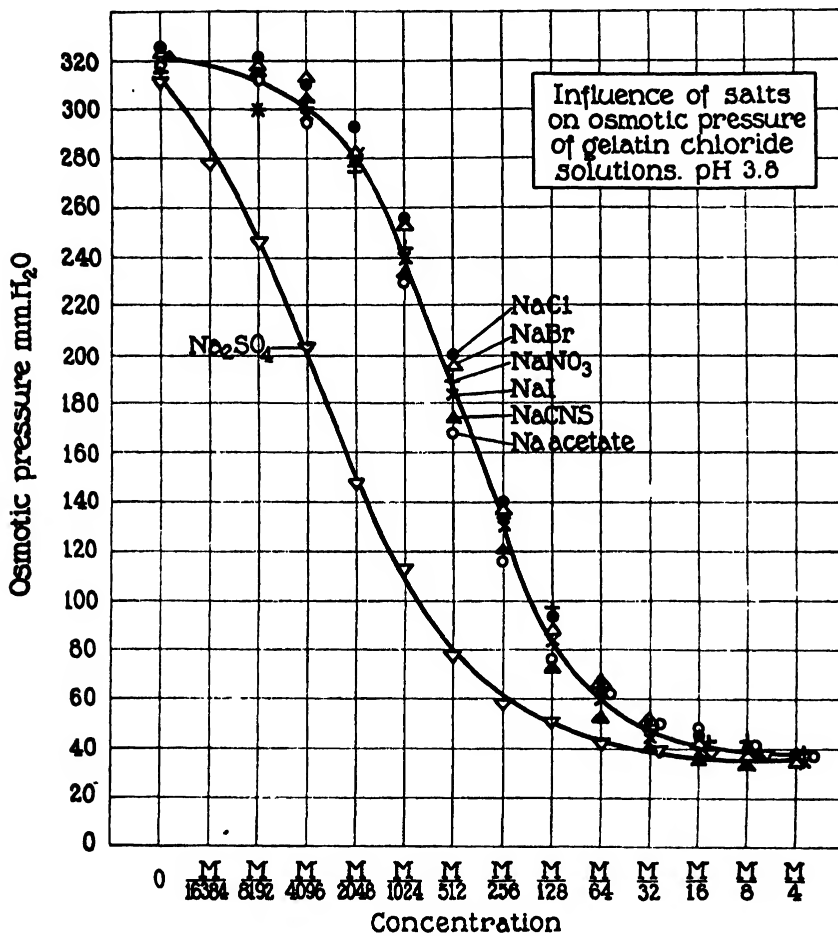

FIG. 3. All salts with monovalent anions depress the osmotic pressure of gelatin chloride solutions of pH 3.8 to the same extent (within the limits of experimental accuracy). Na_2SO_4 depresses considerably more.

originally isoelectric gelatin in 100 cc.) lie, within the limits of experimental accuracy, on one curve, which is entirely different from the curve for the effect of Na_2SO_4. The osmotic pressures are a little

over twice as high when the anion of the salt is monovalent than when it is divalent. The variations in the effects of the six salts with monovalent anion are chance variations, since they are also found when no salt is added, *i.e.* at concentration 0 in Fig. 3.

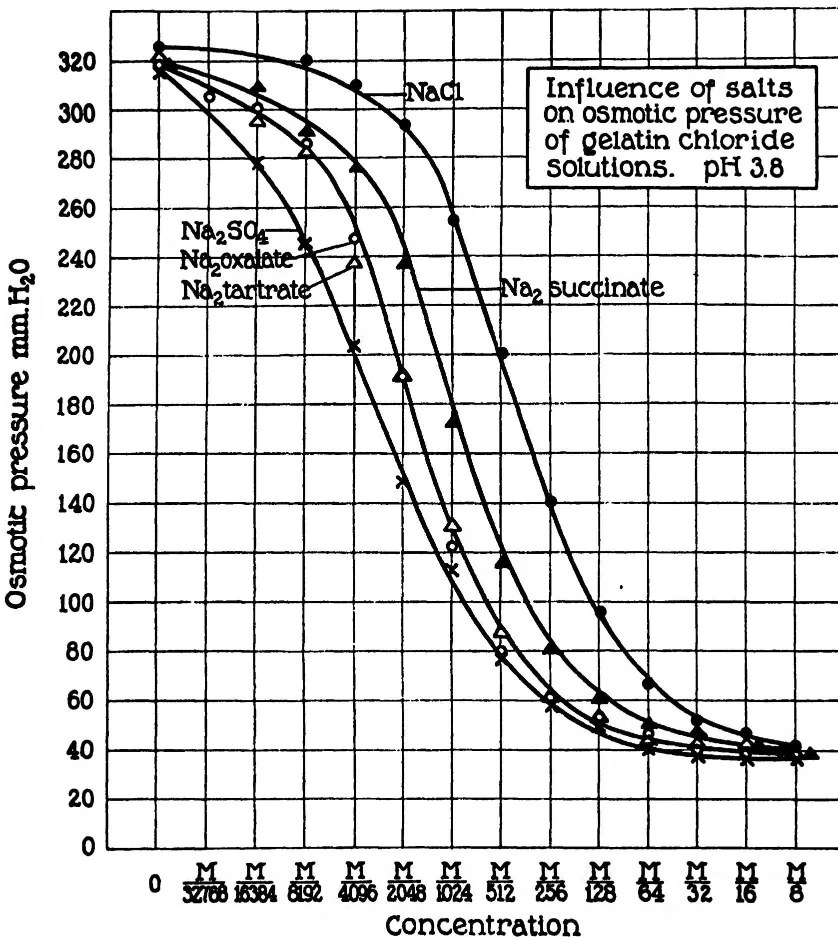

FIG. 4. Depressing effect of weak dibasic and tribasic salts on osmotic pressure of gelatin chloride solution of pH 3.8.

This shows that all the salts with monovalent anions have the same effect on the osmotic pressure when the pH is kept constant and that the Hofmeister anion series is based on error. The anion series has

to be replaced by the valency rule. This statement is supported by Fig. 4 which gives the influence of Na_2 oxalate, Na_2 tartrate, and Na_2 succinate upon the osmotic pressure. The effect of these salts lies between that of Na_2SO_4 and NaCl as the valency rule demands.

Hence we come to the conclusion that when the pH of the solution is kept constant, the influence of the anion of different salts on the osmotic pressure of a gelatin chloride solution is determined by the valency, but not by the chemical nature of the anion. It was shown in former publications that the cation of the salt has no effect on the osmotic pressure of gelatin chloride or edestin chloride solutions. Both the Hofmeister cation and anion series for the influence of salts on the osmotic pressures of protein solutions are fictitious and the consequence of a methodical error.

Swelling.

The general method described in the first part for measuring the influence of acids on swelling was also used for measuring the influence of salts on swelling. It was intended to use gels which at equilibrium had a pH of 3.8. It had been found in previous experiments that 1 gm. of powdered isoelectric gelatin has at equilibrium a pH of 3.82 when it is put into 150 cc. of water containing 4.5 cc. 0.1 N HCl.

The following stock material was prepared: first, doses of wet powdered isoelectric gelatin of 8 gm. each containing about 1 gm. dry weight of isoelectric gelatin; second, a stock solution of HCl containing 3 cc. of 0.1 N HCl per 100 cc. H_2O (since the isoelectric gel of gelatin had in such a solution at equilibrium a pH of 3.82); and third, M/2 solutions of various salts made up with HCl to the same pH as the stock solution of HCl. The stock solution of HCl was used for the dilutions of the M/2 salt solutions.

Doses of 8 gm. of the wet isoelectric gelatin (as described in Part I) were added to 150 cc. of various salt solutions made up as described, and allowed to stand for 2 hours in the solutions at 15°C. The swelling of the gelatin was then measured by weight, as described in Part I.

In the figures the abscissæ are the concentrations of salts, and the ordinates are the weights of the gelatin. Without salts the weight varied generally at the end of the experiment around 27 gm., while it was depressed by salts to about 10 gm.

It had already been shown in preceding papers[2] that when different chlorides, *e.g.* NaCl, $CaCl_2$, or $LaCl_3$, are added to a solution of gelatin chloride, the depressing effect of the three salts on osmotic pressure and membrane potentials is the same for equal concentrations of Cl ions of the salt, provided that the pH is kept constant. This agrees with the theory that the effect of salts is a consequence of membrane equilibria. It seemed of importance to see whether the same is true for the influence of salts on swelling. The influence of LiCl, NaCl, KCl, $CaCl_2$, and $LaCl_3$ was tested and the results are given in Fig. 5. The abscissæ are the molar concentrations of the Cl ions of these salts,

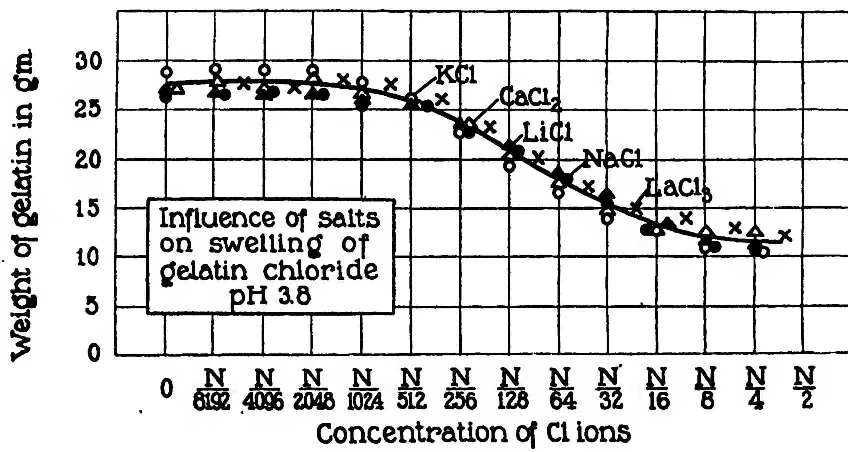

FIG. 5. All chlorides depress the swelling of a gelatin chloride gel of pH 3.8 to the same extent at the same concentration of Cl ions.

while the ordinates are the amount of swelling measured by the weight of the gelatin. It is obvious that the effects of these five salts on swelling lie all on one curve, proving that the effect of salts on swelling of gelatin chloride is determined only by the anion of the salt and that the cation has no effect whatever on the swelling of gelatin chloride.

The next fact to be ascertained was whether or not only the valency of the anion of the salt is of influence or whether the anion series generally quoted in colloidal literature is valid, according to which the swelling is a maximum in NaCNS, and a minimum in Na acetate (leaving the divalent anions out of consideration for the present).

Seven salts with monovalent anions were tried; namely, NaCl, NaBr, NaI, $NaNO_3$, NaCNS, Na acetate, and Na lactate. The results are given in Fig. 6. It is obvious that the effects of all of these seven salts lie on one curve, and that the variations are essentially the chance variations due to the limits of experimental accuracy. This is proven by the fact that the same variations are observed when the concentration of salt is zero; *i.e.*, when no salt is added. There is not the slightest indication of the Hofmeister anion series. Slight influences

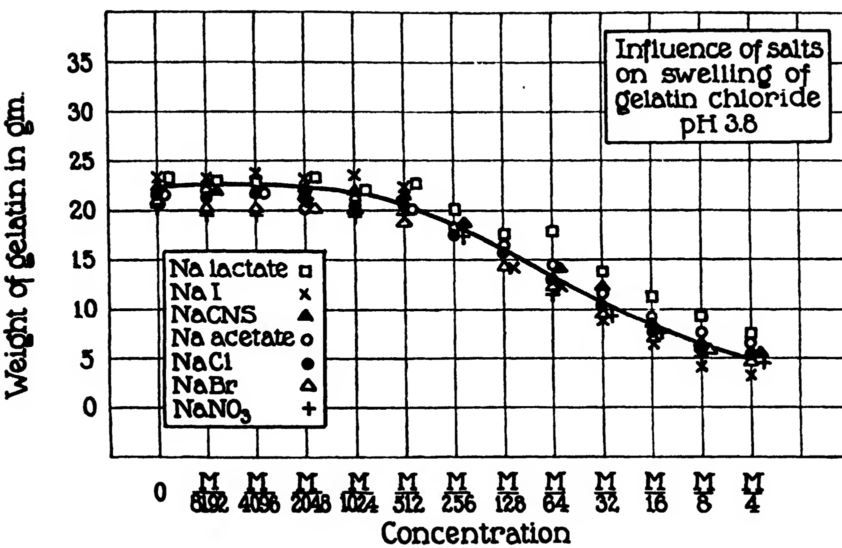

FIG. 6. All salts with monovalent anions depress the swelling of a gelatin chloride gel to the same extent (within the limits of experimental accuracy) at pH 3.8.

of the salts on the cohesion of the gel of gelatin may exist, but they are too small to play a rôle.

While salts with monovalent anions have the same depressing effect for the same concentration of anions, salts with bivalent anions have a much greater depressing effect on swelling than salts with monovalent anions. This is illustrated in Fig. 7 showing the difference in the effect of equal molar concentrations of NaCl and Na_2SO_4 on swelling. NaCl does not depress swelling in concentrations of M/1,024 or below, and the depressing effect of NaCl on the swelling of gelatin chlo-

ride of pH 3.8 commences to be noticeable at a concentration of M/512. This is true for all salts with monovalent anions as Fig. 6 shows. Na_2SO_4 begins, however, to depress at a concentration between M/4,000 and M/2,000, and the curve for the SO_4 effect drops much more rapidly to the minimum than in the case of NaCl.

The effects of salts on the viscosity of gelatin solutions have already been discussed so fully in previous publications by Loeb that it does not seem necessary to add to the facts already published.

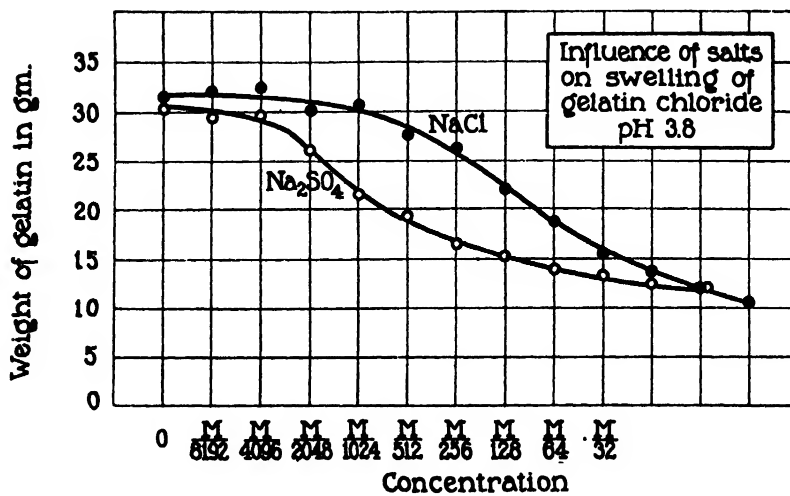

FIG. 7. Na_2SO_4 depresses the swelling of a gelatin chloride gel considerably more than NaCl.

Summarizing all the results, we can say that the membrane potentials, osmotic pressure of gelatin chloride solutions or the swelling of gelatin chloride gels, and that type of viscosity of gelatin chloride solutions which depends on the swelling of submicroscopic solid particles in the solutions are only affected by the anion but not by the cation of a salt; that all anions of the same valency have the same depressing effect on these four properties of gelatin chloride, and that the depressing effect is greater for the divalent than for monovalent anions.

The so called Hofmeister series of the effects of cations as well as of anions on these four properties are fictitious and the result of the

fact that the previous authors have failed to measure the pH of their solutions or of their gels. If this error is avoided and if the pH is kept constant, it is obvious that the Hofmeister series has to be replaced by the valency rule as was to be expected from the membrane equilibria theory of colloidal behavior. This is true only for those four properties of proteins which depend upon membrane equilibria; namely, membrane potentials, osmotic pressure, swelling, and that type of viscosity which depends on swelling. Such properties of proteins as do not depend on the Donnan equilibrium, *e.g.* solubility or cohesion, may be affected not only by the valency but also by the chemical nature of the ions of a salt.

SUMMARY.

It is shown by the older experiments by Loeb and by the experiments reported in this paper that the effect of salts on the membrane potentials, osmotic pressure, swelling of gelatin chloride, and that type of viscosity which is due to the swelling of protein particles, depends only on the valency but not on the chemical nature of the anion of the salt, and that the cation of the salt has no effect on these properties, if the pH of the protein solution or protein gel is not altered by the salt. The so called Hofmeister series of salt effects on these four properties are purely fictitious and due to the failure of the former authors to measure the hydrogen ion concentration of their protein solutions or gels and to compare the effects of salts at the same pH of the protein solution or the protein gel. These results confirm the older experiments of Loeb and together they furnish a further proof for the correctness of the idea that the influence of electrolytes on the four properties of proteins is determined by membrane equilibria. Such properties of proteins which do not depend on membrane equilibria, such as solubility or cohesion, may be affected not only by the valency but also by the chemical nature of the ions of a salt.

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NOTE ON THE PURIFICATION AND PRECIPITATION OF CASEIN.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 16, 1923.)

The numerous methods for the preparation of casein all make use of the fact that casein may be precipitated at its isoelectric point by the addition of acid to an alkaline solution of the protein. It is also known that in the presence of strong acid the protein is "denatured" and becomes permanently insoluble. Great care must be used therefore in the addition of acid. This point was recognized by Van Slyke and Baker,[1] who added the acid slowly with very rapid stirring. The method, however, required very careful handling, and even under the best conditions small amounts of denatured casein are formed, as can be seen from the cloudy solution obtained with alkali. Since it is the acid which causes the formation of this denatured casein, it would appear more logical to make the final precipitation with alkali, since small excess of alkali does not affect the protein. This procedure was tried and was found to yield a product free from denatured casein. The method adopted is briefly as follows.

Casein is precipitated from milk as described by Van Slyke and Baker, except that it is not necessary to take unusual precautions with the addition of acid. The precipitate is thoroughly washed to remove any soluble salts. About 10 gm. of the precipitate are then suspended in a liter of water and the suspension brought to a pH of 2.5 to 3.0 by the addition of HCl. A cloudy solution results. This is then filtered through hardened fluted filter paper, if necessary, several times. A perfectly clear filtrate is obtained which shows only a slight Tyndall cone, and which contains the casein. The filtration is very slow and may require several days, but no other method could

[1] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 127.

be found to remove the very fine particles of denatured protein. This solution is then brought to pH 4.7 by the addition of NaOH. The precipitate of casein is washed several times with distilled water and either dried with acetone or kept as a fine suspension under toluene.

Casein prepared in this way gives an almost water-clear solution in either acid or alkali. If, however, the clear alkaline solution is precipitated by the addition of acid, a milky suspension results which cannot be centrifuged or filtered except in a very narrow range of pH near 4.7. It is possible, therefore, to have two entirely different solutions of casein in the pH range of 5.0 to 7.0 and from 4.5 to 3.0, depending on whether the acid or alkali was added to the precipitate of isoelectric casein or to the solution of casein. The suspension prepared from the precipitate by the addition of alkali consists of a perfectly clear liquid which may be easily filtered or centrifuged from a flaky white precipitate. If the clear alkaline solution is partly precipitated by acid, however, a milky liquid is obtained from which the solid casein cannot be separated by any of the ordinary procedures. This difference is due to the protective action of the casein in solution. As soon as a particle of isoelectric (or denatured) casein is precipitated in the solution, it is covered by a film of the soluble casein salt and therefore the particles do not coalesce. As the isoelectric point is approached, however, the concentration of the soluble casein becomes reduced, and near pH 4.7 becomes so small that it is no longer sufficient to protect the particles which therefore coalesce into large flakes. When, on the other hand, alkali is added to the isoelectric casein, some alkali salt is formed and dissolves. The presence of this salt cannot break up the large flakes of isoelectric casein in suspension, although, as was stated above, it can prevent their formation. The solution of isoelectric casein in alkali is a purely solubility effect, as stated by Loeb,[2] whereas the precipitation of casein from such solutions is complicated by the fact that the solid casein formed is kept in very fine suspension by the protective action of the soluble casein salt.

[2] Loeb, J., and Loeb, R. F., *J. Gen. Physiol.*, 1921–22, iv, 187. Cohn and Hendry (Cohn, E. J., and Hendry, J. L., *J. Gen. Physiol.*, 1922–23, v, 521) have shown conclusively that the solubility of casein in alkali is true solubility governed by the solubility product.

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THE INACTIVATION OF TRYPSIN.

IV. THE ADSORPTION OF TRYPSIN BY CHARCOAL.

BY JOHN H. NORTHROP.

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(Received for publication, May 19, 1923.)

It has been frequently observed that trypsin is more or less completely removed from solution by charcoal. The reaction has been studied in detail by Hedin[1] who found that the amount removed depended on the time of standing, the order in which the components of the solution were mixed, and, in general, behaved as a typical heterogeneous reaction. Hedin also studied the inhibiting action of serum on trypsin and concluded that it was analogous to the action of charcoal. Hussey and the writer[2] have found, however, that the reaction between the inhibiting substance and trypsin could be accurately calculated by the laws of homogeneous reactions. The reaction was found to be completely and instantly reversible and independent of the order in which the components were mixed. The discrepancy between these results and those obtained by Hedin is due to the fact that Hedin allowed the mixture of trypsin and plasma to stand some time before measuring the amount of trypsin combined. Under these conditions the trypsin undergoes a secondary irreversible inactivation which complicates the results. It is quite true that under these conditions the order of mixing, etc., plays an important part, as Hedin found. This is due, however, as the writer showed[3] in the case of the equilibrium between trypsin and the inhibiting substance (formed by its action on proteins), to a secondary irreversible inactivation of the trypsin and is not a property of the reaction between the trypsin and

[1] Hedin, S. G., *J. Physiol.*, 1904–05, xxxii, 390; *Z. physiol. Chem.*, 1906–07, 1497; *Biochem. J.*, 1906, i, 484; 1907, ii, 81; *Ergebn. Physiol.*, 1910, ix, 433.

[2] Hussey, R. G., and Northrop, J. H., *J. Gen. Physiol.*, 1922–23, v, 335.

[3] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 261.

the inhibiting substance. In order to confirm this explanation and to be sure that the reaction with charcoal differs from that with serum and the other inhibiting substances studied, it seemed important to repeat the experiments with charcoal under the same conditions that were used in the experiments with serum. The present paper is a report of these experiments. The results obtained with charcoal confirm those of Hedin, but are qualitatively different from those with serum. There is no analogy therefore between the two reactions and therefore no reason for considering the reaction with serum as heterogeneous.

Experimental Methods.

The trypsin was determined, as described by Hussey and the writer,[4] by noting the time required to cause a 10 per cent change in the viscosity of a standard gelatin solution. The charcoal used was a preparation manufactured by the General Chemical Company and labelled "decolorizing charcoal." It was finely ground and washed with water and acetone before use.

A 2 per cent solution of Fairchild's trypsin in glycerin was used as a stock solution. It was diluted 20 times with water or buffer solution.

The relation between the amount of trypsin removed from solution and the quantity of charcoal is shown in Fig. 1. The curve is of the general form obtained either in adsorption reactions or with reversible homogeneous equilibria. It can be calculated fairly well on either basis but since the adsorption formula is without theoretical interest and the reaction cannot be considered as a reversible equilibrium, it is unnecessary to give the figures. The experiment shows, however, that very little can be deduced from the results of experiments in which the amount of one of the components alone is varied. The results, as a rule, can be calculated either as homogeneous or heterogeneous reactions. The reversibility of the reaction is a much more useful test. Heterogeneous reactions, as a rule, are slowly and *incompletely* reversible, whereas homogeneous equilibria, and especially ionic reactions, are rapidly and completely reversible. As was stated above, the

[4] Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1922–23, v, 353.

reaction between trypsin and the inhibiting substance in serum is rapidly and completely reversible, while the reaction with charcoal is not.

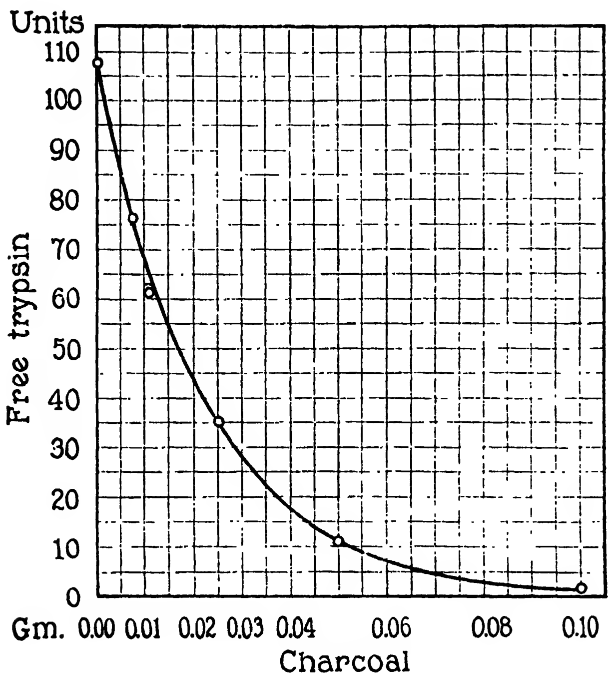

Fig. 1. The effect of increasing amounts of charcoal on the removal of trypsin from solution. 5 cc. of a 0.1 per cent trypsin solution in glycine phosphate acetate buffer pH 8.0 added to the amounts of charcoal indicated. Kept 0.5 hour at 25° C., centrifuged, and trypsin determined in 0.2 cc. of the supernatant liquid.

Effect of the Acidity of the Solution.

The result of an experiment at various pH is given in Table I. The amount of trypsin combined with charcoal is evidently independent of the pH within a wide range. This is entirely different from the results obtained when pepsin combines with solid protein.[5] Unpublished experiments show that trypsin also combines with solid protein and that the combination is markedly affected by the pH.

[5] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 113; 1920–21, iii, 211.

The mechanism is apparently entirely different from the reaction with charcoal and is closely connected with the Donnan equilibrium.

Effect of the Order of Mixing the Components.

The amount of trypsin removed depends entirely on the order in which the components are mixed, as is shown in Table II. If the trypsin and charcoal are mixed first, a very much greater amount of the enzyme is combined than if the charcoal is first mixed with the gelatin. This is typical of a heterogeneous reaction and contrary to most homogeneous equilibria. It is exactly the opposite of the results obtained with serum or other inhibiting substances when the experiment is carried out under the same conditions.

TABLE I.

Effect of the pH on the Adsorption of Trypsin by Charcoal.

5 cc. of glycine phosphate acetate buffer of pH noted + 0.5 cc. of trypsin solution + 0.0125 gm. of charcoal. Kept at 20°C. for 5 minutes, centrifuged, and 0.2 cc. supernatant added to 10 cc. gelatin.

pH	9	7	5	3	7 Control, no charcoal.
Time for 10 per cent change in viscosity, hrs.	0.52	0.49	0.50	0.48	0.15

TABLE II.

Effect of Order of Mixing.

Method of preparing mixture.	Time required for 10 per cent change in viscosity.
	hrs.
1. 1 cc. of H_2O + 0.2 cc. of trypsin, kept 5 min. at 20°C., 10 cc. of gelatin added	0.41
2. 1 cc. of charcoal suspension (≈ 0.1 gm. of charcoal) + 0.2 cc. of trypsin, kept 5 min. at 20°C., 10 cc. of gelatin added	>3.00
3. 10 cc. of gelatin + 1 cc. of charcoal suspension kept 5 min. at 20°C., 0.2 cc. of trypsin added	0.42

The experiment also shows that the combination is not reversible and that the trypsin combined with charcoal is inactive.

It might be supposed that this result is due to the fact that the trypsin is combined with the gelatin in conformation with the usual hypothesis of an intermediate compound between substrate and enzyme. The failure of the trypsin to combine with the charcoal when the enzyme has been previously mixed with gelatin would then be considered as evidence for the existence of such a compound between trypsin and gelatin. This explanation, however, is incorrect as may be seen from Table III, which gives the result of an experiment in which the *charcoal* was previously treated with gelatin. Under these conditions the trypsin is only slightly removed although the charcoal had been washed. The gelatin evidently forms a film on the surface of the charcoal and prevents the trypsin from combining. If trypsin and gelatin combined, it might be expected that the previous treatment of the charcoal with gelatin would increase the amount of trypsin combined instead of decreasing it.

TABLE III.

Effect of Previous Gelatin Treatment of Charcoal on the Adsorption of Trypsin.

	Units of trypsin per 0.2 cc. of solution.
1. 0.1 gm. of charcoal added to 10 cc. of H_2O. Centrifuged, 5 cc. of dilute trypsin added; centrifuged after 5 min. and trypsin determined in 0.2 cc. of supernatant........	<0.10
2. 0.1 gm. of charcoal added to 10 cc. of 3 per cent gelatin pH 7.4. Centrifuged, washed with 10 cc. of H_2O, and trypsin added, etc., as for (1)...	1.0
3. Control, no charcoal........	1.3

SUMMARY.

1. Charcoal removes trypsin from solution. The amount removed depends on the order in which the solutions are mixed. The reaction is not reversible and is almost independent of the pH of the solution.

2. Charcoal which has been previously treated with gelatin does not remove trypsin from solution.

3. The reaction is not analogous either to the reaction between trypsin and the inhibiting substance of serum or to the reaction between solid protein and either pepsin or trypsin.

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THEORY OF REGENERATION BASED ON MASS ACTION.

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(Received for publication, May 15, 1923.)

I.

When a living organism is mutilated, some new growth will generally ensue which would not have occurred without mutilation. Such growth is designated as regeneration for the reason that not infrequently the new growth results in an approximate restoration of the old form of the mutilated organism. Two problems arise; namely, first, to correlate the mutilation with the new growth, and second, to explain the fact that the new growth tends in certain cases towards the restoration of the old form. The two problems are by no means identical though they are often treated as if they were so. Among the older suggestions concerning such a correlation, only that made by Sachs[1] seems of value; namely, that as a consequence of the mutilation, the flow of material in a plant is blocked in the neighborhood of the wound and that this leads to a new growth in the place where the block occurs. This is only part of Sachs' suggestion, the other part being that this material is not the carbohydrates, proteins, or fats, which form the bulk of the living organism, but specific organ-forming substances which he assumed to be as numerous as the morphological differences in the organs; and this was supposed to explain why the new growth tends to restore the old form. These hypothetical specific organ-forming substances, which are assumed to exist in only minute quantities, would today, perhaps, be called hormones. Since a scientific theory must rest on quantitative experiments, which are out of the question as long as the quantities to be measured are as hypothetical as are the specific organ-forming substances of Sachs, this part of his idea has led to no progress. If, however, we disregard

[1] Sachs, J., Stoff und Form der Pflanzenorgane, *Arbeiten des botanischen Instituts in Würzburg, 1878–82*, Leipsic, 1882, ii, 452.

the idea of specific organ-forming substances for the present, focusing our attention on the relation between the measurable quantity of dry matter of the old plant and the quantity of dry matter regenerated in a given time, the law of mass action makes it possible to correlate the mutilation with the process of regeneration. Some of the experiments on which this conclusion is based have already been published[2] and it is intended in this paper to make the proof more complete.

It has long been known that a leaf of *Bryophyllum calycinum* forms new roots and shoots in its notches when it is detached from the plant and kept in moist air or on moist soil. When we determine the dry weight of the roots and shoots produced by such leaves in a given time, it can be shown that they are approximately in direct proportion to the dry weight of the leaves themselves. In order to furnish such a proof, the amount of growth or regeneration must be compared in sister leaves; *i.e.*, in leaves which come from the same node of the same plant. In each node of *Bryophyllum* there are two leaves which have the same age and, as a rule, the same history, and only such leaves are strictly comparable in regard to quantity and efficiency of chlorophyll. We make the assumption that sister leaves, when exposed to equal conditions of illumination, moisture, temperature, and chemical environment will produce approximately equal quantities of products of assimilation in equal times, per gram of dry matter of leaf. This assumption seems to be justifiable on the basis of our present knowledge.

What we can actually prove and have proved already[2] is that equal masses of dry weight of sister leaves, when detached from the plant, produce in equal times under equal conditions of illumination, moisfure, temperature, and chemical environment approximately equal masses of dry weight of roots and shoots from their notches. For the convenience of the readers some new experiments confirming the old results may be described.

Experiment 1.—Seven pairs of sister leaves of equal size were detached from stems and were suspended in an aquarium so that their apices dipped in water (Fig. 1). The experiment lasted from March 20 to April 12, 1923. From the notches near or in the water new roots and shoots arose. It was expected that each leaf of a

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 297, 651.

pair would produce the same mass of roots and shoots from the notches, or that the seven leaves, one each from a pair, would produce the same mass of roots and shoots as their seven sister leaves, per gram dry weight of leaves. This was found to be approximately correct. The roots and shoots were removed from the leaves at the end of the experiment and leaves, roots, and shoots were dried for 24 hours in an electric oven at about 100°C. The two sets of sister leaves are designated as Set I and Set II in Table I.

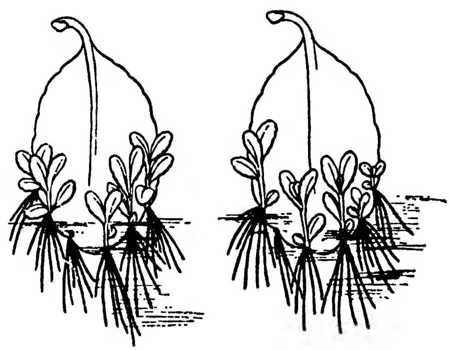

FIG. 1. Isolated sister leaves dipping with their apices into water. March 20 to April 11, 1923. Equal masses of leaves producing equal masses of shoots and roots.

TABLE I.

	Dry weight of leaves.	Dry weight of shoots regenerated.	Dry weight of roots regenerated.	1 gm. dry weight of leaves produced.	
				Shoots.	Roots.
	gm.	gm.	gm.	mg.	mg.
Set I......................	1.528	0.405	0.153	265	100
" II......................	1.665	0.464	0.166	278	100

Each of the two sets of seven leaves produced therefore approximately equal masses of dry weight of shoots and roots per gram dry weight of leaves.

Experiment 2.—Nineteen pairs of sister leaves were used, one leaf of each pair was left intact, while each sister leaf was cut into a small apical and a larger basal piece. All dipped with the apical end into water (Fig. 2). The figure shows that the roots and shoots produced by sister leaves varied approximately in pro-

portion to the mass of the pieces of the leaves. This was confirmed by the measurements in Table II. The experiment lasted from March 26 to April 17, 1923. The nineteen whole leaves are termed Set 2, the nineteen sister leaves each cut into two pieces, 1*a* and 1*b*, are termed Set 1.

TABLE II.

	Dry weight of leaves.	Dry weight of shoots regenerated.	Dry weight of roots regenerated.	1 gm. dry weight of leaves produced.	
				Shoots.	Roots.
	gm.	*gm.*	*gm.*	*mg.*	*mg.*
Set 1, *a*	1.751	0.409	0.095	234	54
b	4.384	0.872	0.248	199	57
" 2	6.060	1.216	0.349	201	58

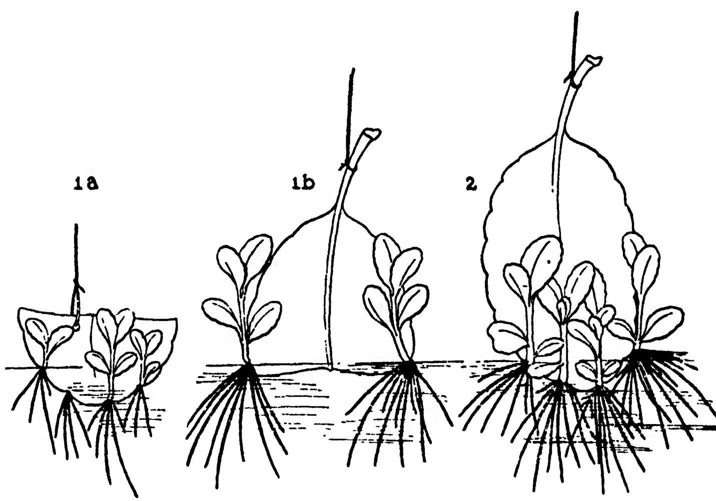

FIG. 2. One leaf (2) left intact, the sister leaf cut into two pieces, a small apical one (1*a*), and a larger basal one (1*b*). Shoot and root production are in proportion to the mass of the pieces. March 26 to April 17, 1923.

The experiment shows that the mass (in dry weight) of shoots and roots regenerated by isolated sister leaves of *Bryophyllum* varies under equal conditions approximately with the mass (in dry weight) of the leaves. On the assumption that the mass of material produced by assimilation in sister leaves, under the influence of light, varies with

the mass of the leaves, it is suggested that the quantity of regeneration is determined by the law of mass action.

That the root and shoot production in the small apical pieces (1*a*) is comparatively a little greater than in the large pieces is probably due to the fact that the sap has not so far to travel to reach the notches

TABLE III.

	Dry weight of leaves.	Dry weight of shoots regenerated.	Dry weight of roots regenerated.	1 gm. dry weight of leaves produced.	
				Shoots.	Roots.
	gm.	*gm.*	*gm.*	*mg.*	*mg.*
Set a_1 (in light)............	3.335	0.838	0.288	251	86
" a (" dark)...........	2.445	0.102	0.008	42	3

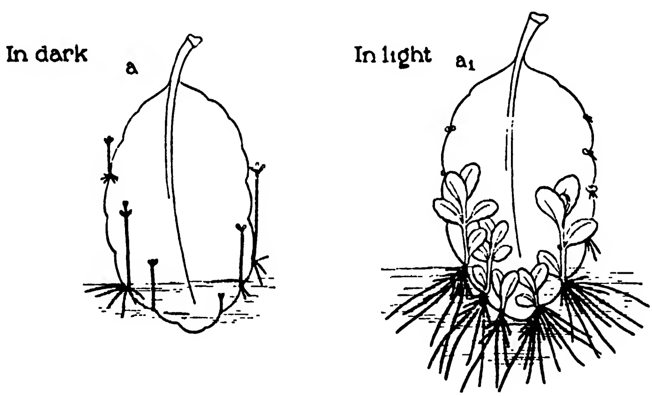

FIG. 3. Influence of light on mass of shoot and root production from detached leaves, one leaf (a) kept in dark, sister leaf (a_1) kept in light. The mass of shoot and root production in the dark is only a small fraction of that in light. March 13 to April 4.

where growth occurs in the small piece (1*a*) as in the larger pieces of leaf (2 and 1*b*).

Experiment 3.—To complete the proof, it is necessary to show that this regeneration depends partly or chiefly on the assimilating effect of the light. Twelve pairs of sister leaves were used, all of which were suspended so that their apices dipped into water as in Fig. 1. One leaf (a_1) of each pair was exposed to scattered daylight, while the twelve sister leaves (a) were kept in the dark but at the same

temperature. The experiment lasted from March 13 to April 4. The total mass of shoots and roots produced in the dark was less than 14 per cent of that produced in the light during the same time (Fig. 3 and Table III).

We may, therefore, draw the conclusion that the quantity of regeneration of a detached leaf of *Bryophyllum* (measured in dry weight of regenerated organs) is determined chiefly by the mass of material produced by assimilation.

Not all the material assimilated in a leaf is, as a rule, used for regeneration. Part of the material is used for the increase of the mass of the leaf. The data proving this fact may be omitted in this paper.

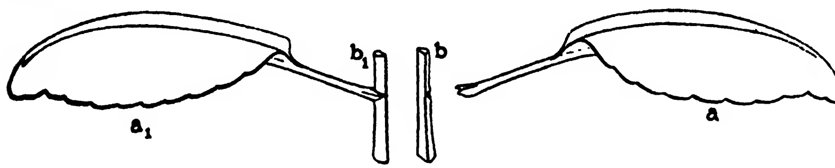

Fig. 4. Method of proving that the dry weight of a piece of stem kept in connection with a leaf increases in weight at the expense of the shoot and root production of the leaf which is correspondingly diminished.

II.

The conclusion that the mass law determines the quantity of regeneration in a leaf enables us to solve the fundamental problem of regeneration; namely, why mutilation leads to new growth where no growth would have occurred without mutilation. This question if adapted to the case of *Bryophyllum* means, why new roots and shoots arise from the notches of a leaf as soon as this leaf is detached from the plant, and why no such growth arises from the leaf as long as it is connected with a healthy plant. The answer is that as long as the leaf is part of a healthy plant, the greater part of the material formed in the leaf by assimilation goes into the stem and is used here for the normal growth of the plant. When the leaf is detached, this material becomes available for new growth (regeneration) of roots and shoots in the notches of the leaf. The method of proving this fact has already been described.[2] Pieces of a stem of *Bryophyllum* containing one node with two leaves each were cut out from a plant. Each piece of stem was split longitudinally through the middle (Fig. 4), to make the

two pieces of half stem (b and b_1) as much alike as possible. In order to minimize the error in cutting, a larger number of pieces of stem were used for one experiment. One half stem (Fig. 4, b), was removed at once from one of each pair of sister leaves, and the dry weight of these half stems (b) was determined immediately. The two sets of sister leaves (one with a half stem attached, the other without a half stem) were suspended for several weeks in moist air, their apices dipping into water. It was found that the leaves (a_1) with a half stem (b_1) attached formed a smaller mass of shoots and roots than the leaves without such a piece of stem. At the end of the experiment, the dry weights of the leaves, shoots, roots, and of the half stems which had been left in connection with the leaves were determined. It was found that the dry weight of the half stems (b_1) left in connection with the leaves (a_1) had increased and that this increase in weight was sufficient to account for the excess in the dry weight of

TABLE IV.

	Dry weight of leaves.	Dry weight of shoots regenerated by leaves.	Dry weight of roots regenerated by leaves.
	gm.	*gm.*	*gm.*
Set I (with stem attached to leaf)...........	2.991	0.427	0.132
" II (without stem)......................	3.116	0.939	0.273

roots and shoots formed in the leaves (a) without pieces of stem. In other words, the inhibitory action of the stem on the regeneration in the leaf was due to the fact that the leaf sent part of its material into the stem, which otherwise would have been available for regeneration in the leaf.

In such experiments the axillary bud of the half stem left in connection with the leaf grows out and it is natural to infer that this growth withdraws material from the stem. It can be shown, however, that the stem inhibits the regenerative growth in the leaf also if this axillary bud is removed at the beginning of the experiment; and that, in this case also, the inhibitory influence of the piece of half stem on regeneration is due to the fact that the leaf sends part of the material produced by assimilation into the stem. In the stem it is used chiefly for callus formation at the basal end and for increase in thickness as well

as for longitudinal growth of the piece of stem (Fig. 5, I). The data in Table IV show the inhibiting effect of the piece of half stem attached to the leaf on the quantity of regeneration in the leaf.

Experiment 4.—Nineteen pairs of sister leaves were used. The pieces of half stem attached to one of each pair of sister leaves were about 25 mm. long.

The total dry weight of regenerated roots and shoots was 559 mg. in Set I and 1,212 mg. in Set II.

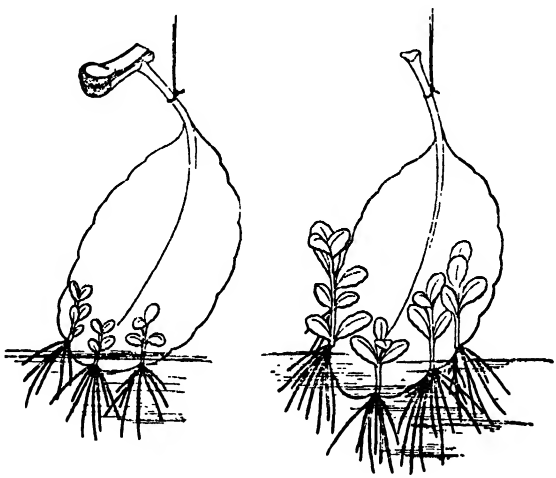

FIG. 5. Leaf I with a small piece of stem attached produces a smaller mass of shoots and roots than the sister leaf II without stem. The piece of stem increases in mass, especially through callus formation, and this accounts for the inhibiting action of the stem on shoot and root formation in leaf. Axillary bud of stem removed. April 6 to April 26.

Hence the presence of the small piece of stem in Set I (Fig. 5) diminished the quantity of regeneration in the nineteen leaves of this set by 653 mg. (The correction for the slight difference in the mass of the two sets of leaves reduces this value to about 630 mg.)

The dry weight of the nineteen half stems determined at the beginning of the experiment was 0.747 gm. The dry weight of the nineteen half stems (which were left in connection with the leaves) determined at the end of the experiment was 1.213 gm. Hence the stems connected with the leaves gained 466 mg. Since this gain

must have occurred through material furnished by the leaf, the inhibitory influence of the stem on the regeneration in the leaf is within the limits of accuracy of the experiments accounted for by the flow of material from the leaf into the stem.

When the axillary bud of the half piece of stem is not cut out, but allowed to grow, the flow of material from the leaf into the stem is

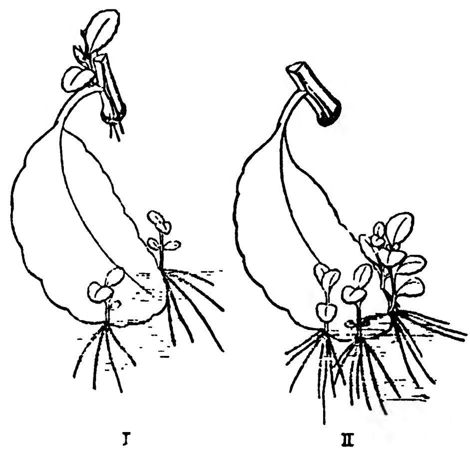

Fig. 6. The inhibitory action of a piece of stem on root and shoot formation in leaf is greater when the axillary shoot can grow out as in I than in sister leaf II where the axillary bud of the stem was removed. March 13 to April 9.

TABLE V.

	Dry weight of leaves.	Dry weight of shoots regenerated by leaves.	Dry weight of roots regenerated by leaves.
	gm.	*gm.*	*gm.*
Set I (with axillary shoot)..................	1.745	0.056	0.027
" II (without axillary bud)................	1.754	0.267	0.068

much more considerable; and, accordingly, the inhibitory influence of the stem on regeneration in the leaf is still more considerable as is shown in the following experiment.

Experiment 5.—Pieces of stem about 25 mm. long with one node each possessing two leaves were cut out and the pieces of stem were split longitudinally as nearly in the middle of the stem as possible (Fig. 6). In this case, none of the pieces of stem were removed; but in one set of leaves the axillary buds of the stem were

removed (Fig. 6, II), while in the other set of leaves the axillary bud was not removed (Fig 6, I). The leaves were suspended in an aquarium, dipping with their apices into water. The axillary shoots grew out in eleven of the stems and these and their sister leaves with stems, the buds of which were cut out, were selected for a measurement of the influence of the growth of the axillary bud on the regeneration in the leaf (Fig. 6). It was found that the inhibitory effect of the half stems on the regeneration in the leaf was greater when the axillary bud of the stem was allowed to grow out (Fig. 6, I) than when this was not the case (Fig. 6, II). The experiment lasted from March 13 to April 10, 1923. Table V gives the quantitative results.

The dry weight of the two sets of leaves was about the same, but the dry weight of the roots and shoots that they produced was considerably smaller when the axillary bud of the stem was allowed to grow than when this was not the case. The leaves of Set I, Fig. 6, the stems of which formed axillary shoots, produced in all only 83 mg. dry weight of roots and shoots, while the leaves of Set II, Fig. 6, the stems of which formed no axillary shoots, produced in all 335 mg. of roots and shoots; *i.e.*, four times as much. This difference is accounted for by the weight of the eleven axillary shoots formed in the stems of Set I, which was 0.454 gm.

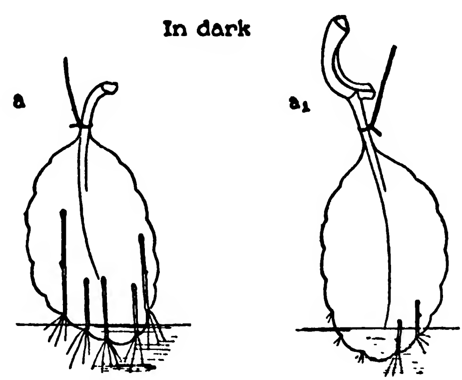

FIG. 7. The inhibitory effect of the stem occurs also in the dark, leaf *a* producing a greater mass of shoots and roots than its sister leaf a_1, which has a piece of stem attached. April 18 to May 9.

In these experiments the piece of stem connected with a leaf was very small, only about 25 mm. When the piece of stem is large, it can absorb more material and as a consequence has a greater inhibiting

power on shoot and root production in a leaf. Figures supporting this statement have already been given and the later drawings in this paper, *e.g.* Figs. 8 to 12, show that leaves when connected with large pieces of stems form no roots and shoots, at least not in the restricted time of these experiments.

In the experiments referred to in this paper the apex of the leaf dipped into water. This is, however, not a necessary prerequisite; what has been said remains true also when the leaves are suspended in moist air.

Experiment 6.—We have seen that the amount of shoot and root formation of a leaf in the dark is only a small fraction of the quantity of regeneration in the light. It was next of interest to find out whether there occurs in the dark, too, a diminution of the shoot formation in the leaf when a piece of stem is attached to it and whether such an inhibition is accompanied in the dark also by a corresponding

TABLE VI.

Dark Experiment.

	Dry weight of leaves.	Dry weight of shoots regenerated by leaves.	Dry weight of roots regenerated by leaves.
	gm.	*gm.*	*gm.*
Set a (nine leaves without stems)...........	1.035	0.070	0.007
" a_1 (" sister leaves with pieces of half stems attached)........................	1.027	0.006	0.004

increase in the dry weight of a stem. It was found that the stems actually diminish or repress the already small amount of shoot formation of a leaf in the dark and that the dry weight of the stems increases to about the same amount in weight as the shoot formation in the leaf is diminished. Fig. 7 indicates the difference in the appearance of the leaves with and without stems attached when kept in the dark. The experiment lasted 20 days. The leaf a, without stem, produced more shoots than its sister leaf a_1 with a half piece of stem attached. The stems gained correspondingly in weight. The exact figures of the dry weight measurements of the experiment are given in Table VI.

The nine leaves (a) without stems, formed in all 77 mg. of shoots and roots, while the sister leaves (a_1) with a piece of half stem attached, formed in all 10 mg. of shoots and roots, a difference of 67 mg. The half stems had at the beginning a dry weight of 0.454 gm., while the half stems of a_1 had at the end of the experiment a dry weight of

0.505 gm., an increase of 51 mg. Hence the half stems in Set a_1 gained in the dark about enough in dry weight to account for the inhibitory effect of the stem of the leaf a_1 on regeneration in the leaf. In this experiment the stems formed no axillary leaf. In a repetition of the same experiment in the dark, in which seventeen pairs of leaves with half stems were used, seven of these leaves, in which the stems remained attached, formed axillary buds. As a consequence more material flowed from the leaves into the stems and the inhibition of shoot and root formation in the leaves with half stems attached became even more complete. Table VII gives the results. The experiment lasted 22 days.

The leaves without half stems produced in all 175 mg. more dry weight of shoots and roots than the leaves with half stems attached in which the inhibition of root and shoot formation was almost

TABLE VII.

Dark Experiment.

	Dry weight of leaves.	Dry weight of shoots regenerated by leaves.	Dry weight of roots regenerated by leaves.
	gm.	*gm.*	*gm.*
Set *a* (seventeen leaves without stems)............	1.740	0.161	0.019
" *b* (" sister leaves with pieces of half stems attached).........................	1.732	0.005	0

complete. The dry weight of seventeen half stems at the beginning was 0.571 gm., at the end 0.879 gm. This latter figure included the weight of seven axillary shoots. Hence the dry weight of the stems increased by 308 mg., more than enough to account for the inhibitory action of the stems on shoot and root production in the leaves with half stems attached.

In these experiments the material sent by the leaf into the stem in the absence of light had been formed previously by the light. In this respect the regeneration of plants in the dark resembles the regeneration in starving animals, where the regeneration depends also on the hydrolysis of stored material.

We now understand why the leaf of *Bryophyllum calycinum*, when it is detached from the plant, forms shoots and roots from its notches,

while this regeneration is inhibited when the leaf forms part of a normal plant. The leaf connected with a normal plant can be dipped into water without forming roots or shoots in its notches. All the material available for shoot and root formation in the leaf is sent into the stem. During a recent visit in Bermuda, I have had a chance to examine thousands of plants of *Bryophyllum calycinum* without finding a single case where a leaf connected with a plant had formed roots or shoots. The same has been true in my greenhouse, and only recently have I had a chance to observe about six plants the older leaves of which formed some tiny shoots. The plants where this occurred were old and in two boxes containing no other plants; so that the suspicion is justified that they had suffered some common injury or disease. When a stem contains many leaves, and when the growth of the stem is stopped or when the sap flow has suffered, it is probable that shoots and roots may originate on leaves still connected with the stem. All that is needed for such growth is that the flow of material from the leaf into the stem should be partially or completely prevented.

The fact that in such cases regeneration can occur in leaves connected with a stem and hence without injury eliminates the idea that "wound hormones" or "wound stimuli" are the cause of shoot and root formation in the notches of a detached leaf of *Bryophyllum*.

This then solves the first part of the problem of regeneration, namely the correlation of the new growth with the mutilation, and the solution is this, that as a consequence of the mutilation the sap and the solutes it contains collect in places where they could not have collected without the mutilation. The quantitative method of experimentation made it possible to prove this correlation.

This correlation holds also for regeneration in the stem. A defoliated piece of stem cut out from a healthy plant of *Bryophyllum calycinum* forms shoots from the most apical buds and roots at the base. In a preceding paper[3] it had been shown that the dry weight of roots and shoots formed in such a defoliated stem increases approximately with the mass of the stem, when the latter is exposed to light. Since the stem contains chlorophyll, this quantitative cor-

[3] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 447.

relation between mass of stem and mass of regenerated roots and shoots suggests that the mass law controls regeneration in this case also. This is supported by the fact that in the dark regeneration of roots and shoots by the stem is as considerably diminished as it is in the case of the regeneration of the leaf.

III.

In the case of a defoliated stem, there enters the phenomenon of polarity inasmuch as shoots are only formed at the apex and roots only at the base of the piece of stem. In this case, regeneration may be said to lead to a semblance of restoration of the original form of the mutilated organism, and this leads us to the second problem of regeneration; namely, to explain the polar character of the regeneration.

Since the mass of roots as well as of shoots regenerated in a piece of stem increases in direct proportion with the mass of the stem, it is obvious that the whole mass of material available for regeneration in the stem must be active in both shoot and root formation. If, nevertheless, shoots are only formed at the apex, one or more additional factors must be responsible for the fact that shoots are not also formed at the base. Two possibilities present themselves: first, the old assumption that there is a chemical difference in the nature of the substances fit for root and shoot formation, the material fit for shoot formation moving only in the ascending current, while the descending current carries root-forming material. A second possibility is that the material of each, ascending as well as descending, sap can give rise to both types of organs, but that the anlagen for root and shoot formation are contained in different histological layers of the stem; the cell layers where roots are formed being normally reached only by the descending sap and the layers where the shoots are formed being normally reached only by the ascending sap.

Specific root- and shoot-forming substances are not yet definitely known to exist, and our knowledge concerning the difference in the location of the vessels for the descending and ascending sap is not sufficiently definite to decide between the two possibilities. It is possible, however, to prove experimentally that the sap sent out by a leaf in the descending stream can accelerate also the rate of growth of

shoots in the more basal parts of the stem, provided such basal shoots are given a chance to grow.

Experiment 7.—In Fig. 8, *a* is a piece of stem without leaf, *b* a piece of stem with a reduced piece of leaf attached to the apex, and *c* a stem with a whole leaf attached to the apex. The right side of the upper part of the stem opposite the leaf is cut off. The stems of *b* and *c* have formed roots at the base, but only on that side of the stem where the leaf is, showing that the material for root formation was carried in the descending current from the leaf. Moreover, the mass of roots is greater

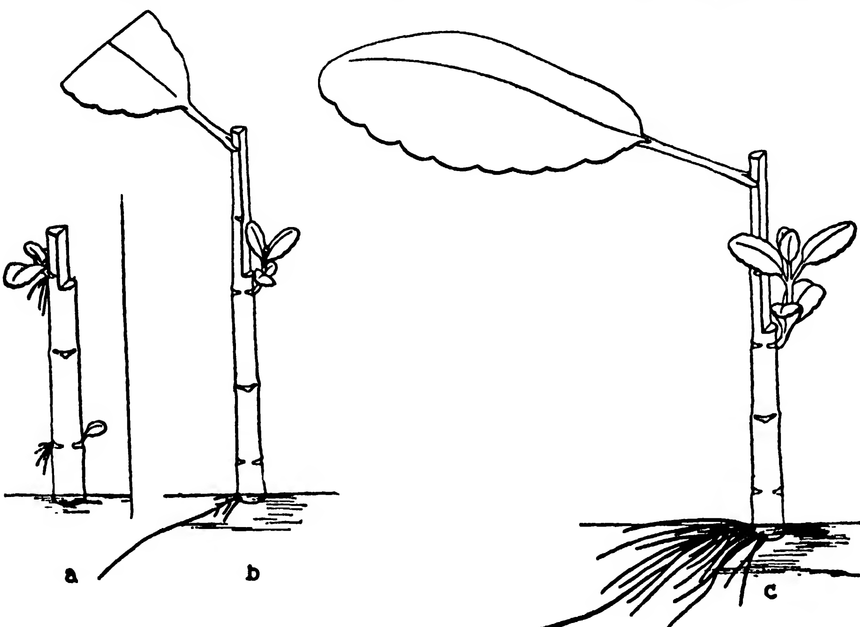

FIG. 8. Proof that the descending sap from a leaf can also under proper conditions increase shoot production. The shoot production in *c* with a whole apical leaf attached is greater than in *b* with only a piece of leaf attached. Without leaf, in *a*, the shoot production is a minimum. Roots at the base form in *b* and *c* first on that side of the stem where the leaf is. December 7 to January 5.

in *c* than in *b* corresponding to the difference in the mass of the leaf. Later on, however, roots may form in the whole circumference of the base of the stem. Stem *a*, which had no leaf, has not formed any roots at the base, but only the transitory air roots in nodes, these air roots disappearing when the permanent roots at the base are formed (as had been shown in a previous publication).[4]

[4] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 687.

All three stems have formed shoots, but the shoot is greatest in *c* where the mass of the leaf is greatest, is smaller in *b* where the leaf is reduced in size, and smallest in *a* where there is no apical leaf. In other words, it seems as if the shoots in *b* and *c* had at least been partly produced from material sent in the descending current from the apical leaf. This inference was supported by quantitative determinations of the dry weight (Table VIII).

All these experiments were carried on simultaneously and lasted from December 7, 1922 to January 5, 1923.

The determinations of the dry weight show that the mass of shoots produced per gram of dry weight of stem increases with the size of the apical leaf, and that therefore the material of which the shoots in *b* and *c* are formed is partly furnished by the descending sap from the leaf.

TABLE VIII.

	Dry weight of stems.	Dry weight of shoots.	Dry weight of basal roots.	Weight of shoots produced per 1 gm. of stem.
	gm.	*gm.*	*gm.*	*mg.*
a (six stems without leaves).............	3.252	0.070	0	21.5
b (four " with reduced leaves)..........	1.883	0.059	0.014	31.0
c (five " " whole leaves)............	3.934	0.180	0.074	46.0

The dry weight of the mass of leaves in *b* was 0.470 gm., in *c*, 2.607 gm.

The increase in the shoots produced from the descending sap from the apical leaf increased with the mass of the apical leaves, but less rapidly. The mass of the basal roots increased, however, almost in direct proportion with the mass of the leaf.

Part of the material sent out by the leaf in the descending current is utilized for the growth in length and thickness of the more peripheral tissues of the stem from which the roots originate, leaving only a fraction of the material of the descending current free to be utilized for the growth of the shoots in *b* and *c* of Fig. 8.

This is supported by the following fact also observed in Experiment 7 that when a leaf is left at the base of a small piece of stem, as in Fig. 9, the stem produces per gram dry weight more apical shoots

than were produced in *b* or *c* of Fig. 8. 1.869 gm. dry weight of stems, each with a basal leaf, produced 0.348 gm. dry weight of apical shoots, or 183 mg. of apical shoots per gm. of stem; in the same time and the same conditions the stem *c*, Fig. 8, produced only 46 mg. dry weight of shoots per gm. of stem when the leaf was at the apex. The dry weight of the basal leaves in Fig. 9 was only slightly more than in *c*; namely, 2.899 gm. instead of 2.607 gm. When the leaf is at the base, the material carried from the leaf to the apical shoot must move in the ascending current to the stem and in this case apparently less material is lost on the way by absorption on the part of the cells of the stem.

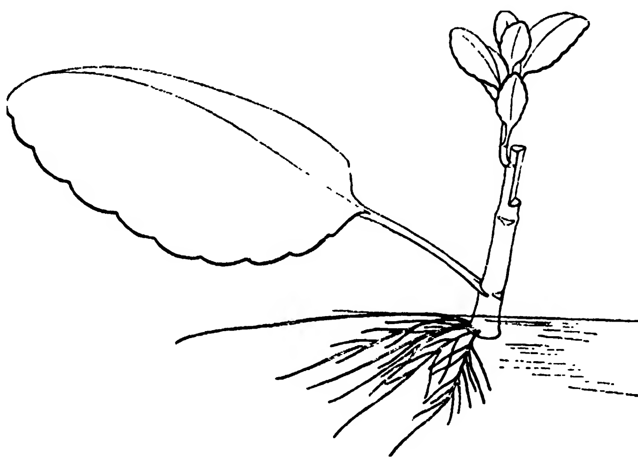

FIG. 9. Basal leaf promotes also shoot production at apex.

That the descending current from a leaf carries material that can be utilized for shoot formation can also be demonstrated in the following form of experiment.

Experiment 8.—In Fig. 10 the two stems *a* and *b* are split longitudinally from the apex down to near the base which dips into water. Stem *a* has no leaf, while stem *b* has an apical leaf to the right. The experiment lasted from January 12 to February 8, 1922. Stem *a*, without a leaf, formed no roots at the base in water, but two tiny shoots at the apex. Stem *b* formed roots at the base on that side only where the leaf is, and a shoot at the apical end of the stem on the opposite side of the leaf. This shoot exceeded in mass the two tiny shoots formed in *a*. The determinations of the dry weight confirmed this (Table IX).

The difference in the mass of shoots produced by *a* and *b* of Fig. 10 can only be ascribed to the material which was carried down from the leaf in the descending current to the base of the stem, rising afterwards on the other side of the stem. The dry weight of the six apical leaves of Set I was 3.388 gm.

In experiments of this kind, the mass of the apical leaf must be large in comparison with the mass of the stem, otherwise too much

TABLE IX.

	Dry weight of stems.	Dry weight of shoots regenerated.	Dry weight of roots regenerated.
	gm.	*gm.*	*gm.*
I (six stems with apical leaf *b*)............	2.802	0.358	0.121
II (five " without leaves *a*)............	2.462	0.044	0.002

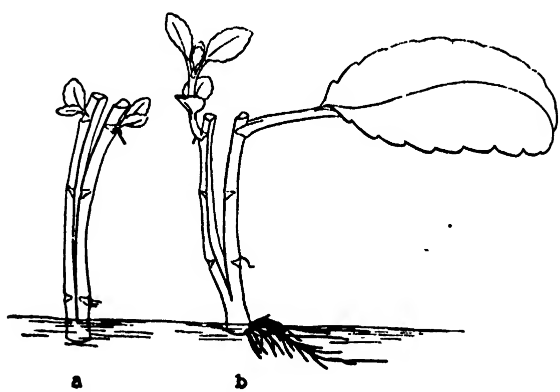

FIG. 10. Stems split longitudinally from apex almost to the base. Stem *b* with one apical leaf forms a shoot with greater mass than the stem *a* without leaf. Root formation in *b* first on side of leaf. January 12 to February 8, 1922.

of the material from the leaf is consumed in the descending current by the cortical tissues of the stem. This was ascertained by special experiments which may here be omitted.

Experiment 9.—Three sets of four small stems each were split longitudinally and suspended horizontally (Fig. 11). Set I had a large leaf at the apex, Set II a reduced leaf, and Set III had no leaf. All produced shoots at the upper apical node, but, as Fig. 11 shows, the size of the shoots increased with the size of the

apical leaf. In this case, the sap from the leaf had to travel in the descending current to the base of the stem and then on the other side of the split back to the apex. There can be no doubt that the descending current from the apical leaf favored shoot formation. In Set I, 1 gm. dry weight of stem produced 57 mg. dry weight of shoot, in Set II, 37 mg., and in Set III, which had no leaf, only 12 mg.

An interesting modification of the experiment is that represented in Fig. 12, *a*. A piece of stem with one leaf left at the apex was split lengthwise at some distance beneath the leaf, until near the base, which dipped into water. The apical bud opposite the leaf was

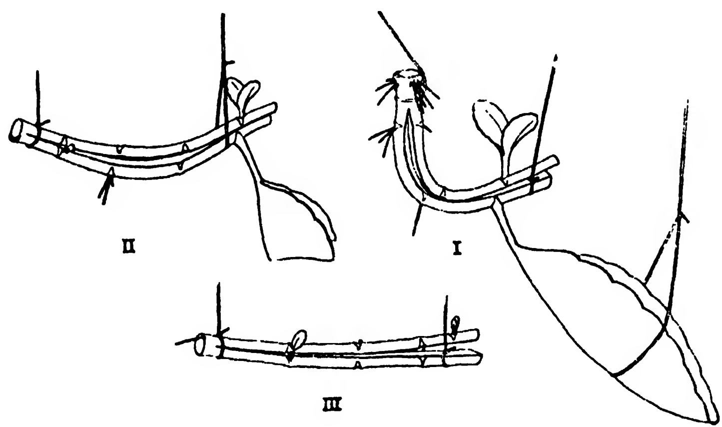

FIG. 11. Similar experiments as in Fig. 10, except that stems and leaves are suspended horizontally in moist air. I, with whole leaf, forms a larger shoot at apex than II with a leaf reduced in size. III, without leaf, produces only tiny shoots. Notice also that geotropic curvature of stem increases with mass of leaf. April 3 to April 21, 1923.

removed as indicated diagrammatically in the figure. In this case the descending sap had to flow down the stem on the side opposite the leaf and on this side the first roots developed at the base, spreading finally, however, all around the base. No shoot developed on that side (Fig. 12, *a*). A shoot developed, however, on the same side where the leaf is, at the apex of the split part of the stem (Fig. 12, *a*). Now the question arose, whether or not the descending sap of the leaf contributed to the growth of this shoot. This turned out to be true as the following quantitative experiment shows.

Five stems without leaves were split lengthwise down to near the base, as shown in Fig. 12, *b*, each split half producing a shoot at the apex. Six stems of almost the same mass but with one leaf left at the apex, as in Fig. 12, *a*, were split as indicated (Fig. 12, *a*). The latter stems produced one shoot at the apex of the split half of the stem as indicated in Fig. 12, *a*, and occasionally a second smaller shoot in the node below. Now the total dry weight of shoots produced by the six stems in *a* was 1.557 gm., while the total dry weight of shoots produced by the five stems in *b* without leaves was only 0.668 gm. The dry

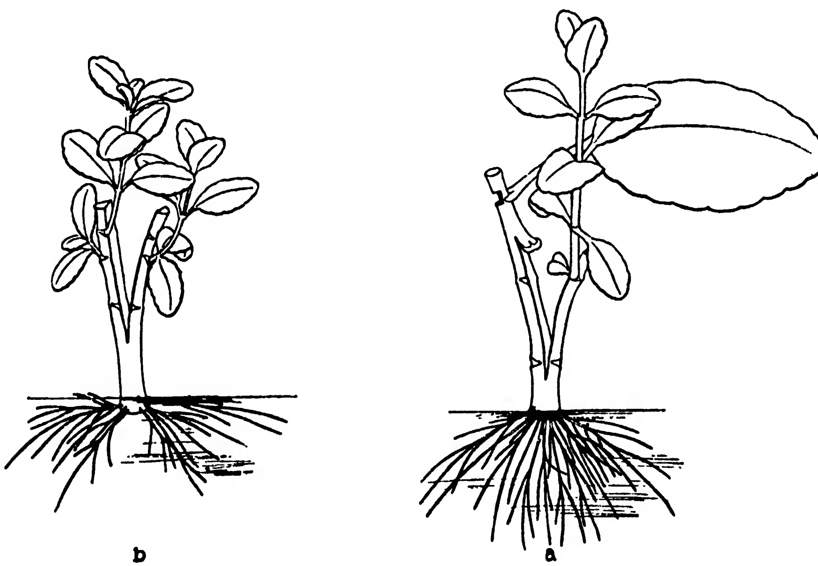

FIG. 12. Stem with leaf attached produces a greater mass of shoots than stem without leaf. January 9 to March 16, 1923.

weight of the six apical leaves was 2.063 gm., about the same as that of the stems. This leaves no doubt that the descending sap from the apical leaf contributed to the growth of the shoots below the leaf in *a*. In this case, the descending sap had to travel down the whole length of the stem on the side opposite the leaf (Fig. 12, *a*), and had to then ascend again to the apex of the split part of the stem. The exact figures are given in Table X. The mass of regenerated shoots is so great because the experiment lasted longer than usual; namely, over 2 months (January 9 to March 16).

These and other experiments leave no doubt that the sap sent out by a leaf in the descending current through the stem contains material fit for shoot production. According to Sachs the descending current should carry only root-forming substances and this makes it difficult to explain the second problem of regeneration—which, in this case, is the polar character of regeneration—on the assumption that the descending sap carries only specific root-forming substances. That the sap of the leaf is fit to give rise to both roots and shoots is also demonstrated by the fact that in each notch of an isolated leaf roots as well as shoots grow out and that the rate of growth of both occurs in proportion to the mass of the leaf.

This compels us to consider the other possibility; namely, that in the stem the anlagen for roots and shoots are more widely separated than in the leaf and that the descending sap in the stem reaches primarily only the root-forming tissues of the stem.

TABLE X.

	Dry weight of stems.	Dry weight of shoots regenerated.	Dry weight of roots regenerated.
	gm.	*gm.*	*gm.*
a (six stems with apical leaf)...............	2.139	1.557	0.306
b (five " without leaves)...............	1.987	0.668	0.040

V.

SUMMARY AND CONCLUSIONS.

1. The writer's older experiment, proving that equal masses of isolated sister leaves of *Bryophyllum* regenerate under equal conditions and in equal time equal masses (in dry weight) of shoots and roots, is confirmed. It is shown that in the dark this regeneration is reduced to a small fraction of that observed in light.

2. The writer's former observation is confirmed, that when a piece of stem inhibits or diminishes the regeneration in a leaf, the dry weight of the stem increases by as much or more than the weight by which the regeneration in the leaf is diminished. It is shown that this is also true when the axillary bud in the stem is removed or when the regeneration occurs in the dark.

3. These facts show that the regeneration of an isolated leaf of *Bryophyllum* is determined by the mass of material available or formed in the leaf during the experiment and that such a growth does not occur in a leaf connected with a normal plant for the reason that in the latter case the material available or formed in the leaf flows into the stem where it is consumed for normal growth.

4. It is shown that the sap sent out by a leaf in the descending current of a stem is capable of increasing also the rate of growth of shoots in the basal parts of the leaf when the sap has an opportunity to reach the anlagen for such shoots.

5. The fact that a defoliated piece of stem forms normally no shoots in its basal part therefore demands an explanation of the polar character of regeneration which lays no or less emphasis on the chemical difference between ascending and descending sap than does Sachs' theory of specific root- or shoot-forming substances (though such substances may in reality exist), but which uses as a basis the general mass relation as expressed in the first three statements of this summary.

6. It is suggested that the polar character of the regeneration in a stem of *Bryophyllum* is primarily due to the fact that the descending sap reaches normally only the root-forming tissues at the base of the stem, while the ascending sap reaches normally only the shoot-forming anlagen at the apex of the stem.

7. This suggestion is supported by the fact that when the anlagen for shoots and roots are close together as they are in the notch of a leaf, the sap of the leaf causes the growth of both roots and shoots from the same notch and the influence of the sap of the leaf on this growth increases for both roots and shoots in proportion with the mass of the leaf.

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THEORY OF GEOTROPISM BASED ON MASS ACTION.

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From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 19, 1923.)

In a previous publication the writer had shown that when a piece of stem of *Bryophyllum* is cut out from a plant and suspended horizontally in a moist atmosphere, the stem bends, the lower side becoming convex, the upper concave. He was able to prove that this geotropic curvature is a phenomenon of growth, the cortical tissues of the lower side of the stem growing more rapidly in length than the cortical tissues on the upper side of the stem or than the tissues of the wood. He was also able to show that the geotropic curvature is more rapid when the piece of stem possesses one or two apical leaves than when it is free from leaves.[1]

If growth is a function of mass action, then this geotropic curvature of the stem must have its cause in the fact that a greater mass of the material required for growth must collect on the lower than on the upper side of a stem suspended horizontally. This can happen if the liquid—the tissue fluid—containing this material collects in greater quantity on the lower than on the upper side of the stem, or, in other words, if this tissue fluid follows gravity. In order to test the idea that geotropic curvature is a function of chemical mass action, it is necessary to find out, first, whether the mass of material sent by a leaf into a stem increases with the mass of the leaf, and second, whether the geotropic curvature of a stem suspended horizontally increases also with the mass of the leaf.

First, a simple experiment proves that a piece of stem gains more in dry weight when it is attached to a large than when it is attached to a small piece of leaf. Fifteen pieces of stem, each about 25 mm. long, with one node at the apex and a pair of leaves in the node were split longitudinally, and as accurately as possible in the middle,

[1] Loeb, J., *Bot. Gaz.*, 1917, lxiii, 25.

so that the two pieces of stem had approximately equal masses. The leaf of one half stem was left intact, while that of the other was reduced in size (Fig. 1). Both leaves dipped into water. Fig. 1 shows the appearance of the stems after 12 days. The pieces of stem attached to a large leaf had formed a callus and were geotropically bent, while the others had undergone no or little geotropic curvature and showed no or only traces of callus formation. At the end of the experiment, which lasted from April 13 to April 28, 1923, the dry weight of the whole leaves was 2.573 gm. and that of the reduced

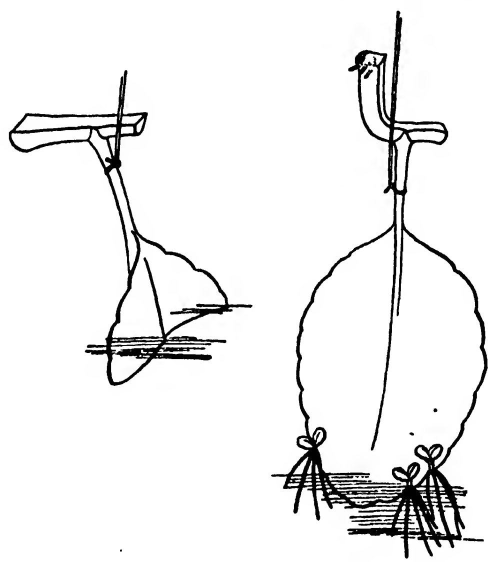

Fig. 1. Influence of mass of leaf on callus formation and geotropic curvature of stem. April 13 to April 25.

leaves 1.083 gm. The dry weight of the half stems attached to the whole leaves was 0.868 gm. while that of the half stems attached to the reduced leaves was only 0.765 gm. Hence the larger leaves sent 103 mg. more material (by dry weight) into the stems than did the smaller leaves. This mass of material which goes from the leaf into the stem is here used for callus formation and for that growth which gives rise to geotropic curvature when the stem is suspended horizontally. The next problem was to find out more accurately whether or not the curvature of a stem suspended horizontally increases with the mass of the apical leaf.

In such experiments it is necessary to remember that the geotropic curvature of a stem is the resultant of two opposing forces. One is the excess of longitudinal growth of cortical tissue on the lower side of the stem suspended horizontally over that of the rest of the stem; the opposing force is the rigidity of the upper layers of the stem, chiefly the wood. When the wood is too hard, the stem cannot bend. The influence of equal masses of an apical leaf on the rate of geotropic curvature of two stems can only be equal if the rigidity of the wood is identical in the two stems, a condition which cannot often be fulfilled. Quantitative work of this kind must therefore be statistical; but it is only intended here to prove the validity of the mass law for geotropism in a semiquantitative way. It is necessary to select for experimentation only the more apical parts of the stems of young plants where the wood is still soft, or not too rigid, otherwise little or no curvature is possible. The method of the experiments is illustrated in Fig. 2 of this paper. Stems of about equal flexibility were selected and defoliated with the exception of one leaf at the apex. With the aid of a string around the petiole of the leaf, the stem was suspended in a moist aquarium. In order to secure a horizontal position of the stem, the latter was put on a wire netting bent in the shape of a U on the bottom of which the stem was allowed to rest (Fig. 2). The meshes of the net were $\frac{1}{2}$ inch and the squares of the wire netting made it easy to follow and measure the slope of geotropic curvature from day to day. It was found that under proper conditions this curvature increases with the mass of the leaf as intimated in Fig. 2. Three stems (*a*, *b*, *c*) were selected. each having a leaf at the apex. The leaves in *b* and *c* were partly cut off so that the mass of the leaves in *a*, *b*, and *c* was roughly in the ratio of $1:\frac{1}{2}:\frac{1}{4}$. The drawing was made after 11 days. The figure shows that the degree of geotropic curvature in the three stems increased with the mass of the apical leaf, being a minimum in *c* and a maximum in *a*. When the stem contains no leaf, the curvature is a very slow process.

It seemed more accurate to modify the experiment by always comparing the rate of curvature of two halves of a stem split longitudinally as in Fig. 3, *a* and *b*. A stem with a pair of apical leaves was split as accurately as possible in the middle between the two leaves and each half was suspended as shown in the figure. The

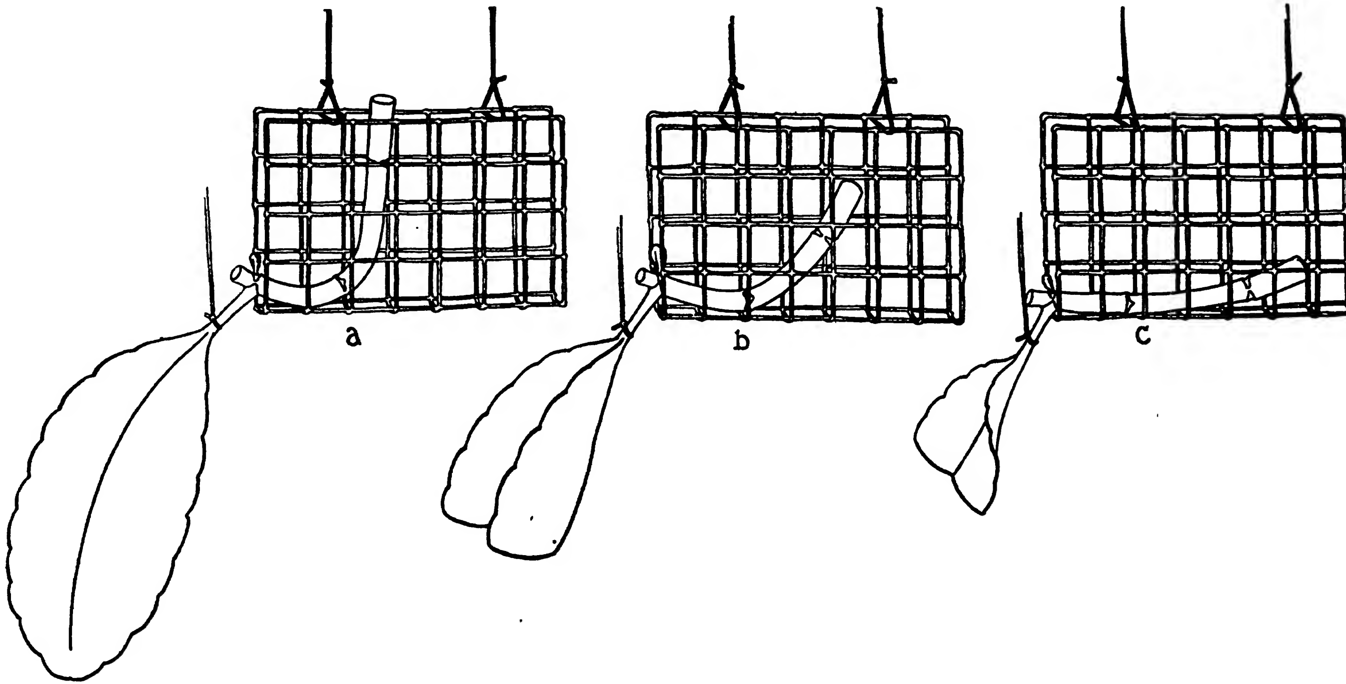

FIG. 2. Influence of mass of leaf on rate of curvature, *a* whole leaf, *b* half leaf, *c* about ¼ of a leaf. Curvature is greatest in *a*, least in *c*. April 27 to May 8.

leaf was on the lower side of the stem as in the preceding experiment, but while one leaf remained intact, part of the other was cut off, so that its mass was only about one-half of that of the whole leaf. Fig. 3 gives the curvature after 3 days, the curvature being greater where the mass of the leaf was greater. Fig. 4 gives the curvature of the two half stems a day later, and Fig. 5 on the 6th day.

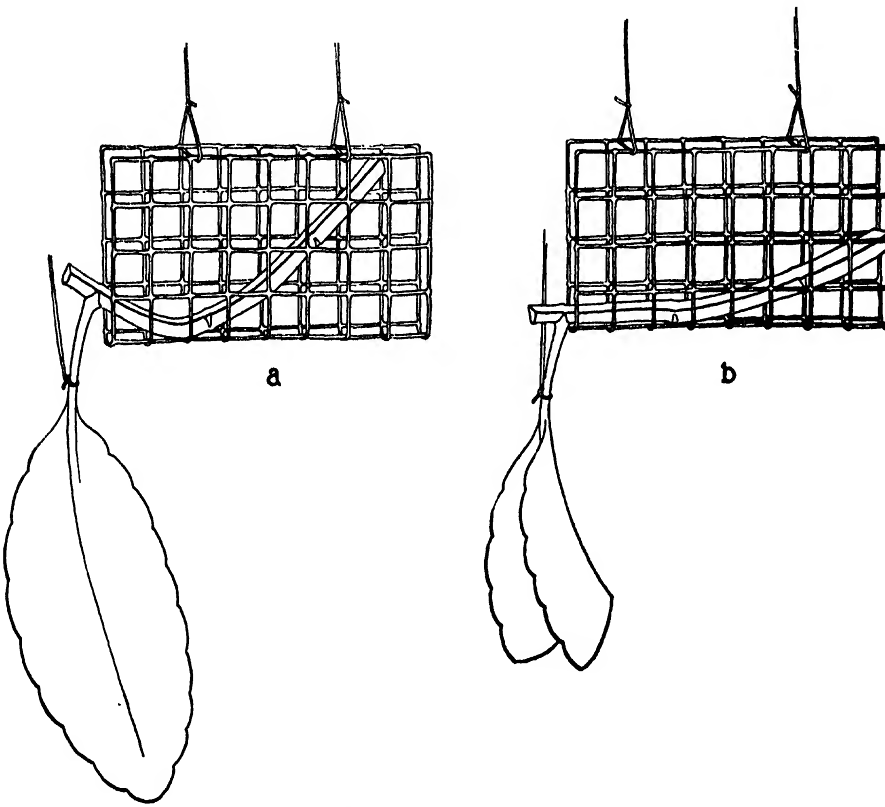

FIG. 3. Stem split lengthwise, one piece having a whole the other only half of a leaf. Curvature is greater in whole leaf. May 8 to May 11.

This experiment gives also an idea of the rapidity with which the curvature occurs at the temperature of the greenhouse (about 24°C.).

It may finally be of interest to show that the rate of geotropic curvature of these half stems with an apical leaf attached occurred approximately in proportion with the mass of the leaf. In Fig. 6

the curvature of three half stems each with a whole leaf was compared with the curvature of the three sister halves each with a reduced leaf. All the three stems with whole leaves had reached about the same curvature as stem *a*, and therefore this one alone is reproduced here. *b*, *c*, and *d* had bent approximately in proportion with the mass of the apical leaf attached.

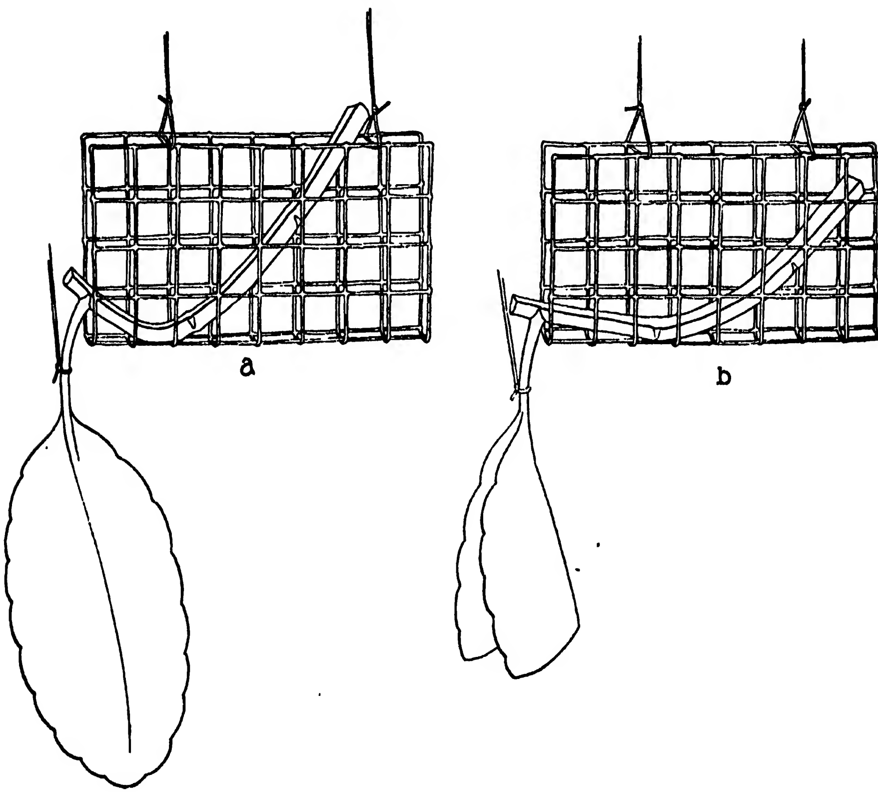

Fig. 4. The same specimens as in Fig. 3. May 12.

In Fig. 7 a stem was split longitudinally and both halves were suspended horizontally. One half had a leaf at the apex, while the other half had no leaf. The latter bent very slowly in comparison with the half which had an apical leaf attached, in accordance with the difference in the mass of material available for growth in the two half stems. It may be stated incidentally that the mass of shoots

and roots regenerated by the half stem with a leaf was also considerably larger than the mass of shoots and roots formed in the half stem without leaf. The experiment lasted from April 10 to May 23.

The experiments with split stems give on the whole less reliable results than those with whole stems. Not only the unavoidable

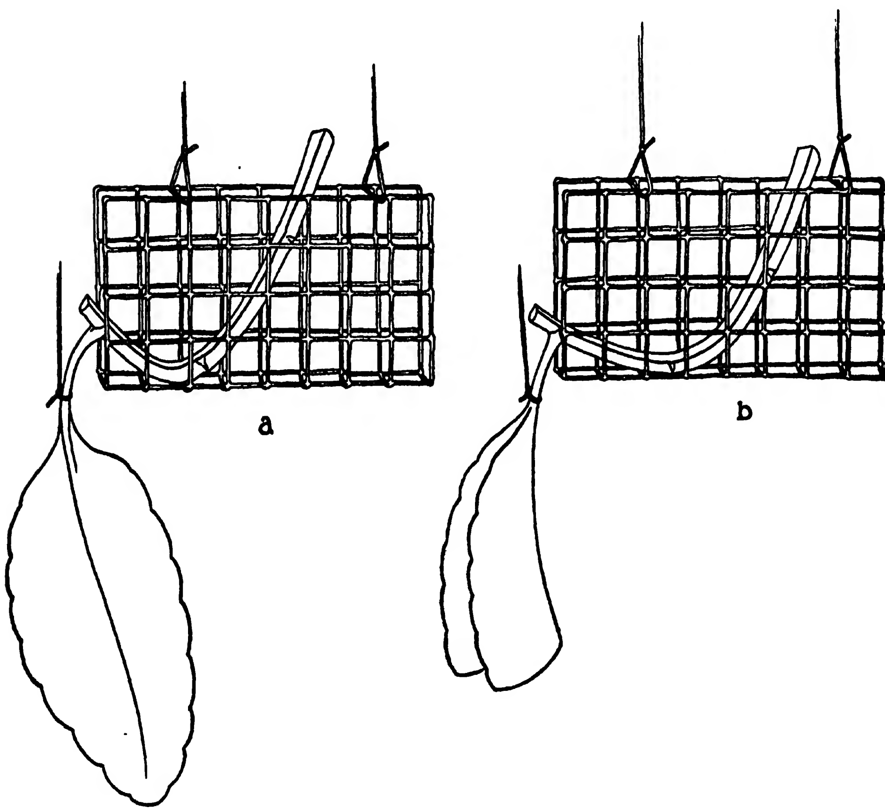

FIG. 5. The same specimens as in Figs. 3 and 4. May 14. Rate of curvature increases with mass of leaf.

errors in halving the stem, but possibly other variables vitiate the result, *e.g.* the unequal degree of drying of the upper side of the stem and the resulting inequalities in rigidity of the wood.

In all these experiments the leaf was at the apex of a piece of stem. When the leaf is at the base, no geotropic curvature ensues. This can be best demonstrated on stems split longitudinally which have

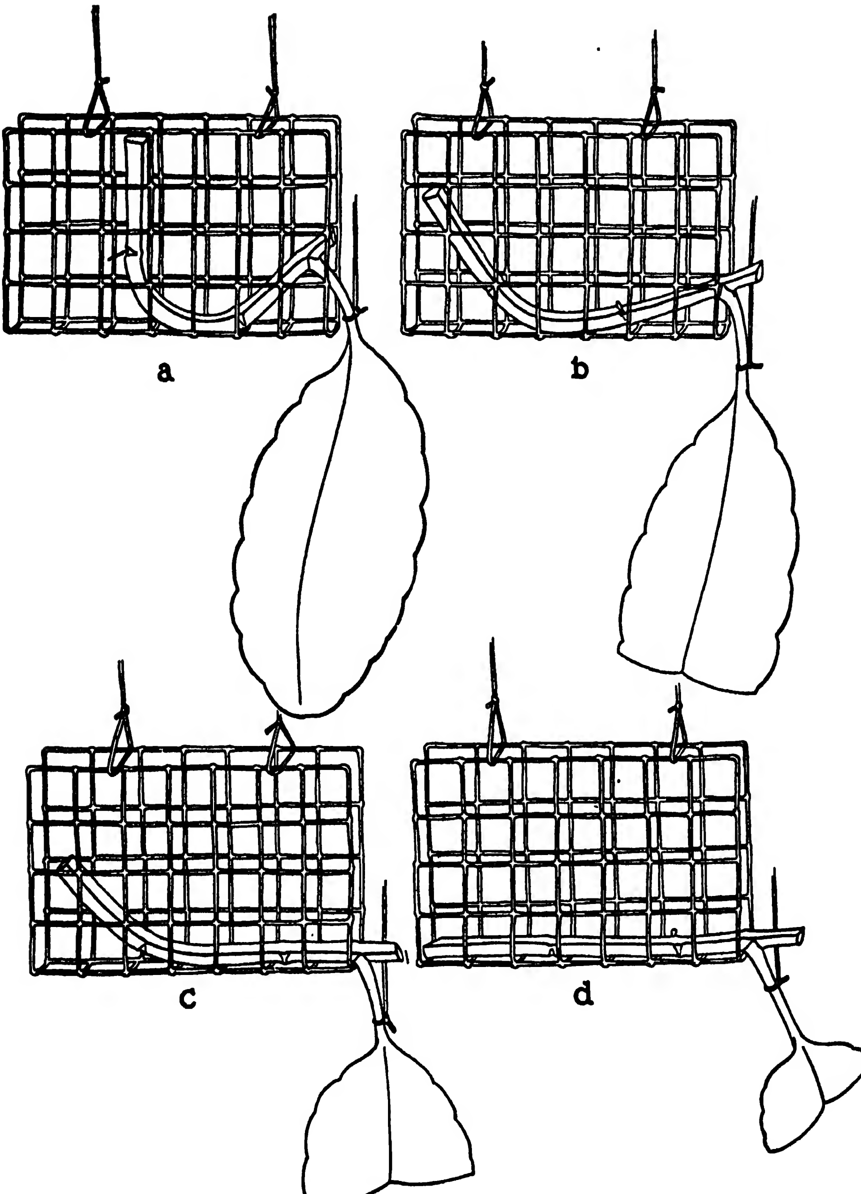

FIG. 6. *a*, piece of stem with whole leaf; *b*, *c*, and *d* with increasingly reduced leaf. The geotropic curvature increases with mass of leaf. May 8 to May 17.

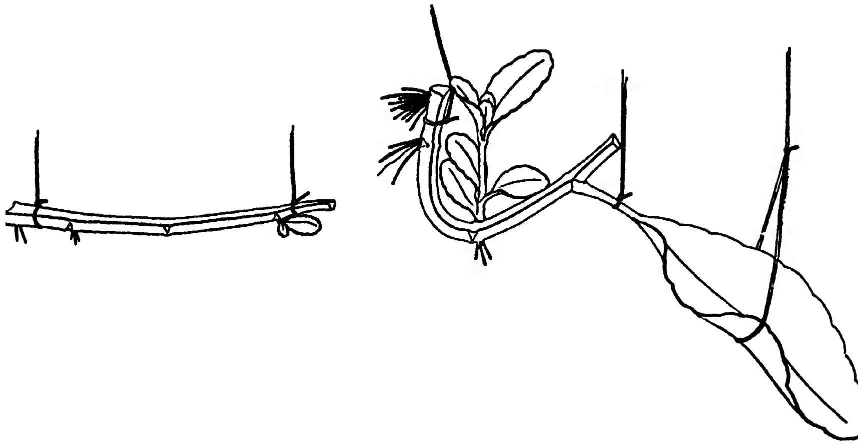

FIG. 7. Stem split longitudinally and suspended horizontally, one half is entirely defoliated, the other has a leaf attached to the apex. The latter bent rapidly in the usual way, while the half stem without leaf bent only very slowly. April 10 to May 23.

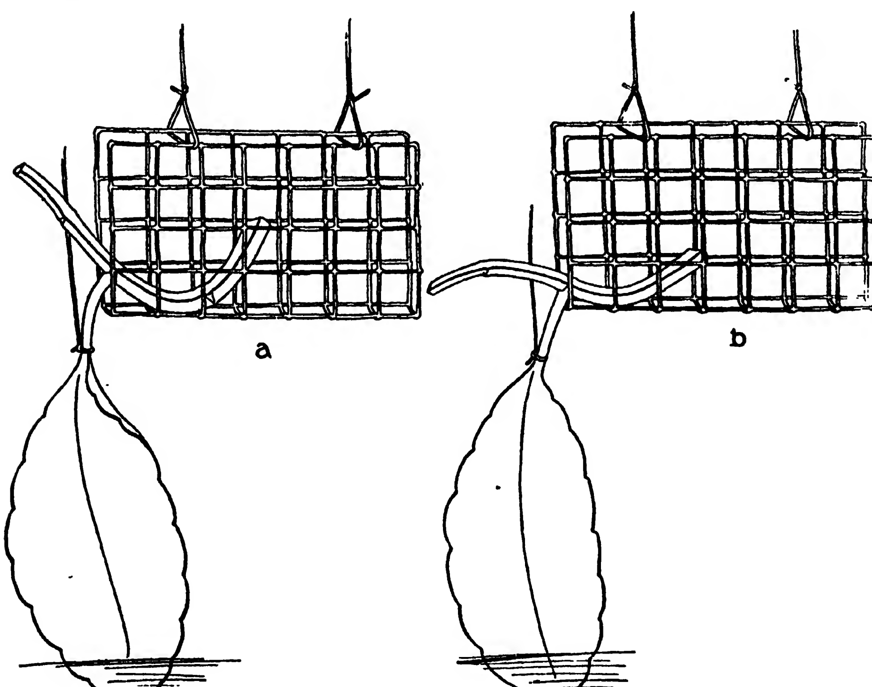

FIG. 8. Leaf in middle of stem. Geotropic curvature only in basal part of stem; the apical piece of stem shows no curvature or one in the opposite sense. May 14 to May 16.

a leaf in the middle of the stem as in Fig. 8. In 2 days the part basal to the leaf showed the typical geotropic curvature; the part of the stem apical to the leaf did not bend. There was, perhaps, a tendency to bend in the opposite direction but the writer is not certain that this curvature is connected with growth. This and many similar experiments on split or whole pieces of stem confirmed the fact that the leaf accelerates the geotropic curvature only in a basal piece of stem. This agrees with the idea suggested in the preceding paper[2] that the descending sap from a leaf reaches the cortical layers of tissue which give rise to root formation, callus formation, and incidentally to that longitudinal growth of the stem which causes geotropic curvature, while the ascending sap reaches only the most apical bud, causing it to grow into a shoot. Geotropic curvature of a horizontal stem is therefore accelerated only by a leaf when it is at the apical but not when it is at the basal end of the stem.

When the apical leaf is on the upper side of a piece of stem placed horizontally, it causes also geotropic curvature, though the rate of bending is probably a little smaller than when the leaf is situated on the lower side of the stem.

The theory of geotropic curvature of the stem of *Bryophyllum calycinum* when put in a horizontal position is then as follows. This curvature is determined by the excess of longitudinal growth of the cortical layers on the under side of the stem and this excess of growth increases with the mass of an apical leaf attached to the stem. The geotropic curvature is therefore a phenomenon of mass action of the material sent by the leaf into the stem or formed in the stem itself.

The fact that the material sent by an apical leaf into the stem collects in greater quantity on the lower side of a stem suspended horizontally may find its explanation in the assumption that under the influence of gravity the tissue sap collects on the lower side of such a stem.

SUMMARY.

1. It is shown that the rate of geotropic curvature of a piece of stem of *Bryophyllum calycinum* when suspended horizontally increases with the mass of an apical leaf attached to the stem.

[2] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 831.

2. It is shown that the dry weight of the stem increases with the mass of the leaf attached and also that the degree of curvature increases with this increase in the dry weight of the stem.

3. The conclusion is drawn that geotropic curvature is in this case a function of mass action of the material sent by the leaf into the basal part of the stem.

4. The material sent by a leaf into the apical part of a stem does not lead to the same geotropic curvature.

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THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS.

By M. HEIDELBERGER, Ph.D., and O. T. AVERY, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 19, 1923.)

In 1917 Dochez and Avery (1) showed that whenever pneumococci are grown in fluid media, there is present in the cultural fluid a substance which precipitates specifically in antipneumococcus serum of the homologous type. This soluble substance is demonstrable in culture filtrates during the initial growth phase of the organisms; that is, during the period of their maximum rate of multiplication when little or no cell death or disintegration is occurring. The formation of this soluble specific material by pneumococci on growth *in vitro* suggested the probability of an analogous substance being formed on growth of the organism in the animal body. Examination of the blood and urine of experimentally infected animals gave proof of the presence of this substance in considerable quantities in the body fluids following intraperitoneal infection with pneumococcus. In other words, this soluble material elaborated at the focus of the disease readily diffuses throughout the body, is taken up in the blood, passes the kidney, and appears in the urine unchanged in specificity. Similarly, a study of the serum of patients suffering from lobar pneumonia has revealed a substance of like nature in the circulating blood during the course of the disease in man. Furthermore, examination of the urine of patients having pneumonia due to pneumococci of Types I, II, and III has shown the presence of this substance in some stage of the disease in approximately two-thirds of the cases. Recently from filtered alkaline extracts of pulverized bacteria of several varieties, including pneumococci, Zinsser and Parker (2) have prepared substances which appear free from coagulable protein. These substances, called "residue antigens,"

are specifically precipitable by homologous antisera. These observers consider these acid- and heat-resistant antigenic materials analogous to the soluble specific substance of pneumococcus described by Dochez and Avery (1). In spite of the fact that these "residue antigens" are precipitable by homologous sera produced by immunization with the whole bacteria, Zinsser and Parker have so far failed to produce antibodies in animals by injecting the residues.

In the earlier studies by Dochez and Avery certain facts were ascertained concerning the chemical characteristics of this substance. It was found that the specific substance is not destroyed by boiling; that it is readily soluble in water, and precipitable by acetone, alcohol, and ether; that it is precipitated by colloidal iron, and does not dialyze through parchment; and that the serological reactions of the substance are not affected by proteolytic digestion by trypsin. Since the substance is easily soluble, thermostable, and type-specific in the highest degree, it seemed an ideal basis for the beginning of a study of the relation between bacterial specificity and chemical constitution. The present report deals with the work done in this direction.

EXPERIMENTAL.

The organism used in the present work was Pneumococcus Type II. The most abundant source of the soluble specific substance appeared to be an 8 day autolyzed broth culture; hence this material was used as the principal source of supply. For comparison dissolved pneumococci and lots of urine containing the specific substance were also worked up, with essentially the same results, as will be seen from Table I.

The process for the isolation of the soluble specific substance consisted in concentration of the broth, precipitation with alcohol, repeated re-solution and reprecipitation, followed by a careful series of fractional precipitations with alcohol or acetone after acidification of the solution with acetic acid, and, finally, repeated fractional precipitation with ammonium sulfate and dialysis of the aqueous solution of the active fractions.

Five lots of 15 liters each of 8 day cultures of Pneumococcus Type II in meat infusion phosphate broth are each concentrated on the water bath in large evapo-

rating dishes to 1,000 to 1,200 cc. and precipitated in a separatory funnel by the gradual addition, with vigorous rotation, of 1.2 volumes of 95 per cent alcohol. The mixture separates into two layers, and is allowed to stand over night, or for several hours. The upper layer, which is almost black and comprises the largest part of the mixture, contains only traces of the soluble specific substance, and is siphoned off and discarded. The lower, more viscous layer is run into a 250 cc. centrifuge bottle (occasionally a second will be required), capped, and rotated at high speed for ½ hour. Three layers are formed, of which the uppermost is merely a further amount of the liquid previously discarded. The middle layer consists of a compact, greenish cake of insoluble matter and gummy material, and contains most of the soluble specific substance. The bottom layer, from which salts often separate, is a brownish syrup rich in salts and nitrogenous matter and relatively poor in specific substance, and can, by careful manipulation, be poured off to a large extent. Although a small proportion of the specific substance is lost if this syrup is discarded, its elimination represents so considerable a purification as to warrant the sacrifice of the active material contained. The gummy cake remaining in the centrifuge bottle, together with adhering salts and syrup, is now rinsed out and ultimately combined with similar material from the other lots. All of this is then dissolved as completely as possible in water, care being taken to break up the many lumps of gummy material, diluted to 1 liter, and again precipitated with alcohol. In this case about 1.3 liters are required to precipitate all but the last traces of active material from the upper layer. This is again discarded and the lower layer treated as before. At this stage there is relatively less of the bottom layer, and it is more difficult to separate it from the cake containing the specific substance, but as much as possible is removed. The remaining material is smoothed out with water, diluted to about 500 cc., and centrifuged. The precipitate is washed twice with water, and the washings are combined with the main solution. The still turbid liquid, the volume of which should be about 750 cc., is put through the alcohol purification process a third time, about 1.1 liters of alcohol being required. After having been centrifuged, the active material is again dissolved in water, made definitely acid to litmus with acetic acid, and again centrifuged. The precipitate is washed three times with water acidulated with acetic acid, and the filtrate and washings are combined in a separatory funnel and diluted again if necessary to 750 cc. Acetone (redistilled) is now added until a permanent precipitate forms, about 250 cc. being necessary. The precipitate is allowed to settle, whereupon the lower part of the mixture containing the precipitate is drawn off and centrifuged. The clear supernatant fluid is restored to the main solution, while the precipitate, which consists largely of insoluble material and gives an aqueous solution almost devoid of activity, is discarded. Fractional precipitation is continued, and even when the specific substance appears in quantity in the precipitate, it is occasionally possible to separate a lower, inactive, syrupy layer, as in the previous purifications by alcohol. Addition of acetone is continued until a test portion, heated on the water bath to remove acetone, diluted with saline, and neutralized, no longer gives a pre-

cipitate with immune serum, after which the upper layer may be discarded. The active precipitates are then redissolved in water, centrifuged again, and the supernatant liquid is diluted to 375 cc., reacidified with acetic acid, and again fractionated with acetone. If inactive fractions are obtained, the process is again repeated until no further purification results. Alcohol may be used for these fractionations instead of acetone, the only difference being that a somewhat larger proportion is required. The active material is then dissolved in about 150 cc. of water and again made definitely acid with acetic acid. The solution is treated with solid ammonium sulfate until the first slight precipitate forms. This is generally inactive, and if so, may be discarded. Finally, ammonium sulfate is added to saturation, completely precipitating the specific substance if the volume of the solution is not too great. The mixture is allowed to stand for several hours and is then centrifuged and the precipitate washed with a little saturated ammonium sulfate solution. It is redissolved in about 75 cc. of water acidified with acetic acid, centrifuged if necessary, and again precipitated by saturation with ammonium sulfate. Finally, the specific substance so obtained is dissolved in water and dialyzed first against running tap water in the presence of chloroform and toluene, and finally against distilled water until tests for sulfate and phosphate ion are negative. Addition of acetic acid during the early stages of the dialysis assists in the removal of calcium, which otherwise forms a large part of the ash.

The dialyzed solution is concentrated to dryness on the water bath and the residue redissolved in hot water. If the solution is not perfectly clear, it is centrifuged again before being evaporated to dryness, and the whole process is repeated as long as insoluble material separates. Toward the end of the final concentration absolute alcohol may be added to assist in the precipitation of the substance.

Variations in the exact volumes given are often necessary with different lots of broth, but this will occasion little difficulty if all fractionations are controlled by the specific precipitin test.

As so obtained the soluble specific substance forms an almost colorless varnish-like mass which may be broken up and dried to constant weight at 100°C. *in vacuo*. The yield from 75 liters of broth averages about 1 gm., although it varies within rather wide limits in individual lots.

By the method outlined above all substances precipitable with phosphotungstic acid or capable of giving the biuret reaction were eliminated. The residual material (Preparation 17, in Table I), for which no claim of purity is made, as efforts at its further purification are still under way, contained, on the ash-free basis, 1.2 per cent of nitrogen. It was essentially a polysaccharide, as shown by the formation of 79 per cent of reducing sugars on hydrolysis, and by the isolation and identification of glucosazone from the products of hydrolysis.

0.4 gm. of Preparation 17 was heated under a reflux condenser with 40 cc. of 0.5 N HCl for 7 hours. The filtered solution was treated with 0.4 cc. of phenylhydrazine, followed by saturated sodium acetate solution until Congo red paper was no longer turned blue. After 1 hour in the water bath the crystalline osazone was filtered off, washed with water, purified by stirring well with dry methyl alcohol, and again filtered. 0.037 gm. was obtained, melting and decomposing at 196–197°C. with preliminary darkening and softening. A second fraction of 0.026 gm. of even purer osazone was obtained by heating the mother liquors of the first fraction 2 hours longer after addition of 0.2 cc. more of phenylhydrazine and purifying the crude product with methyl alcohol. This portion melted quite sharply at 205–206°. The true melting point of pure glucosazone is 208°.

50 mg. of the combined fractions, dissolved in 3 cc. of alcohol and 2 cc. of pyridine, gave, in a tube 0.5 dm. long, an initial rotation of −0.20° and a final rotation of −0.08°, while Levene and La Forge (3) give −0.62° and −0.35° for a solution of twice the concentration of recrystallized glucosazone. The only other hexose whose osazone rotates to the left is altrose, and this is known only as a laboratory product. Moreover, its osazone melts at 178° and decomposes at 189°.[1]

The aqueous solution of the substance gave the Molisch reaction out to the limit of delicacy of the test. Reduction of Fehling's solution occurred only after hydrolysis. Phosphorus was present only in traces; sulfur and pentoses were absent. A 1 per cent solution gave no biuret reaction, no precipitate with phosphotungstic acid, mercuric chloride, or neutral lead acetate, precipitated heavily with basic lead acetate, and gave a faint turbidity with tannic acid. Calcium is very tenaciously retained, but does not appear to be an essential part of the molecule, as the specific reaction was also given by calcium-free preparations. No color is given by iodine solution.

The soluble specific substance is remarkably stable to acids. A solution in 0.5 N hydrochloric acid maintained its activity undiminished and failed to reduce Fehling's solution after 36 hours at room temperature, but showed reducing sugars and absence of precipitation with immune serum after transfer to the water bath.

The limits of delicacy of the specific precipitation of the soluble substance were titrated, as shown in Table I, and it is seen that Preparation 17 still gave a specific reaction in a dilution as high as 1:3,000,000.

Table I represents a summary of the reactions of some of the earlier preparations worked with, as well as the later ones. Prep-

[1] We are greatly indebted to Dr. P. A. Levene for valuable aid and advice on this phase of the work.

aration 4 was obtained from the urine of a patient with a Type II pneumococcus infection, while No. 8 was obtained from an antiformin solution of the pneumococci. In both of these cases, as well as in Nos. 9, 11, and 15, the method of purification given above had not been fully worked out.

Attempts to stimulate antibody production by the immunization of animals with the purified substance yielded negative results.

TABLE I.

Summary of the Properties of Various Preparations of the Soluble Specific Substance of Pneumococcus Type II.

Preparation No.	Total N.	Hydrolysis.			S	P	Specific rotation $[\alpha]_D$	Precipitation with immune serum.*	Molisch reaction.†
		NH_3 N	NH_2 N	Reducing sugars.					
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*			
4‡	6.1	3.5			1.5		Too dark.	1:80,000	
4A§	4.7			63.0		1.0‖	−20.6°	1:640,000	1:320,000
8¶	2.9			++			+19.8°	1:1,250,000	1:640,000
9	6.6				1.8		−8.6°	1:640,000	
11	2.1	0.9	1.3				+31.6°	1:2,500,000	1:1,250,000
15	2.0			49.0		0.9‖	+30.8°	1:2,500,000	1:1,250,000
17	1.2	C = 46.2 per cent.	H = 6.1 per cent.	79.0	None.	Tr.	+55.7°	1:3,000,000	1:1,500,000

* After 2 hours at 37°C. and over night at 4°.

† Unless the α-naphthol solution is fresh, other colors will mask the purple at high dilutions. The figures represent the dilution of the preparation itself; in other words, the highest dilution giving the reaction in the previous column.

‡ From urine.

§ Preparation 4 repurified.

‖ Due to incomplete dialysis.

¶ From dissolved pneumococci.

DISCUSSION.

While it has long been known that the capsular material of many microorganisms consists, at least in part, of carbohydrates (4), any connection between this carbohydrate material and the specificity relationships of bacteria appears to have remained unsuspected. While it cannot be said that the present work establishes

this relationship, it certainly points in this direction. Evidence in favor of the probable carbohydrate nature of the soluble specific substance is the increase in specific activity with reduction of the nitrogen content, the increase in optical rotation with increase in specific activity, the parallelism between the Molisch reaction and specific activity, the high yield of reducing sugars on hydrolysis, and the actual isolation of glucosazone from a small quantity of the material. The small amounts of substance available up to the present have hindered the solution of the problem, and it is hoped that efforts at further purification of the soluble specific substance, now in progress with larger amounts of material, will definitely settle the question.

SUMMARY.

1. A method is given for the concentration and purification of the soluble specific substance of the pneumococcus.

2. The material obtained by this method is shown to consist mainly of a carbohydrate which appears to be a polysaccharide built up of glucose molecules.

3. Whether the soluble specific substance is actually the polysaccharide, or occurs merely associated with it, is still undecided, although the evidence points in the direction of the former possibility.

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IMMUNOLOGICAL RELATIONSHIPS OF CELL CONSTITUENTS OF PNEUMOCOCCUS.

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(Received for publication, March 19, 1923.)

In the preceding paper (1) observations on the nature of the soluble specific substance of pneumococcus have been recorded. The present work concerns itself with the facts thus far ascertained in a comparative study of the chemical and immunological reactions of the protein[1] of pneumococcus. It seemed of interest to study the immunological relationship of the bacterial protein to the soluble specific substance of pneumococcus, and in the present paper certain differences in the serological specificity of the two classes of substances are brought out and related to their chemical nature. It would be beyond the scope of the present paper to attempt a review of the extensive literature on the subject of bacterial nucleoproteins in general, and this has already been done in reference books (2).

This work is presented, despite its incompleteness, because it points the way to a comparative study of the immunochemical relations existing between two different cellular constituents of the same organism. The first of these components, the so called soluble specific substance, has been discussed in the preceding paper; it need only be pointed out here that this reactive substance possesses none of the chemical properties of protein, that although antigenically it appears capable of stimulating little or no antibody response, serologically it exhibits to an extraordinary degree the reactions of type specificity in antipneumococcus sera. In other words, this non-protein constituent in isolated form is relatively and perhaps

[1] The word protein as used in this paper refers only to that portion of the dissolved pneumococcus cell precipitable in the cold by acetic acid and consisting mainly of nucleoprotein and mucoid.

absolutely inert as antigen, although it is highly reactive in the antibacterial serum of the homologous type of pneumococcus. On the other hand, by the methods described it is possible to separate from the bacterial cell another substance which is protein in nature and is also distinctive in its serological behavior from the soluble specific substance previously discussed.

Although the investigation of the relationship of chemical constitution to biologic specificity of pneumococcus is still in progress, the results thus far obtained are sufficiently interesting and perhaps significant enough to justify the presentation of facts already available.

EXPERIMENTAL.

Method.—The bacteria from actively growing broth cultures of pneumococci are recovered by centrifugation. The amount of material that can conveniently be worked up at any one time is, therefore, dependent on facilities for rapid centrifuging. In the present study broth cultures in units of from 6 to 12 liters have been treated in the following manner. The bacterial sediment, after removal of the culture fluid, is resuspended in about one-tenth volume of 0.85 per cent salt solution. To this suspension is added the minimum amount of bile necessary to effect solution of the bacterial cells. This procedure is carried out at ice box temperature (4°C.) in order to inhibit the possible proteolytic action of the intracellular enzymes which are released by lysis of the cells. Undissolved material is removed from the bile solution by centrifugation; the clear supernatant fluid containing the dissolved constituents of the cells is then treated by the method ordinarily employed for the isolation of the so called nucleoproteins from alkaline extracts of bacteria. The protein is precipitated from solution by the addition of dilute acetic acid (10 per cent). The acid is added slowly and the mixture carefully shaken; flocculation occurs promptly and is complete at a reaction faintly acid to litmus.

The precipitate obtained in this manner is separated by centrifugation and thoroughly washed two or three times in equal volumes of distilled water. The washed precipitate is redissolved in water by adding 0.1 N NaOH until the solution is faintly alkaline to litmus. This process of precipitation and washing is repeated at least three times. Before the last precipitation, the solution is passed through a Berkefeld filter V. The final precipitate is washed rapidly twice with acetone and once with dry ether and dried in a vacuum desiccator. The preparation obtained in this manner and referred to in the text as "protein" is a whitish, dry powder, readily soluble in faintly alkaline solutions.

Solutions of this substance give the usual qualitative color reactions characteristic of proteins of this nature: positive biuret, Hopkins-

Cole, Millon, xanthoproteic, and Molisch reactions. The hydrolyzed protein gives the purine reaction with Fehling's solution. The nitrogen content of a representative specimen of the protein was 16.0 per cent, the phosphorus content 0.5 per cent.

TABLE I.

Precipitin Reactions of Pneumococcus Protein (Type II) and Soluble Specific Substance (Type II).

Protein from Pneumococcus Type II.	Antipneumococcus serum.			Antityphoid Serum "T – A – B."	Normal horse serum.
	Type I.	Type II.	Type III.		
Lot 4, 1:1,000	++	++	++	–	–
" 5, 1:1,000	++±	++	++	–	–
Soluble specific substance of Pneumococcus Type II, 1:50,000.	–	++++	–	–	–

± indicates faint cloud; +, cloud; ++, marked cloud; +++, marked cloud with precipitation; and ++++, heavy precipitation, supernatant fluid clear.

TABLE II.

Precipitin Reactions of Pneumococcus Protein (Type II).

Protein from Pneumococcus Type II.	Antipneumococcus serum.			Normal horse serum.
	Type I.	Type II.	Type III.	
Lot 7, 1:200	++	++	++	–
1:400	++	++	++	–
1:800	++±	++	++	–
1:1,600	++±	+	+	–
1:3,200	++	+	+	–
1:6,400	+	±	±	–

Precipitin Reactions of Protein from Pneumococcus Type II in Antipneumococcus Sera Types I, II, and III.

Clear solutions of protein derived from Pneumococcus Type II were prepared by grinding weighed amounts of dry preparation with water, adding dropwise the minimum amount of 0.1 N sodium hydroxide to effect solution, and diluting to volume with salt solution. The antipneumococcus and antityphoid sera used were diag-

nostic sera prepared by immunization of horses with the respective bacteria. The reactions were carried out by adding increasing dilutions of protein solution in amounts of 0.5 cc. to an equal volume of serum so diluted that each 0.5 cc. contained 0.2 cc. of the original serum. All tests were incubated in the water bath at 37°C. and readings made at the end of the incubation period and after 18 hours at ice box temperature.

From Tables I and II it appears that solutions of protein prepared from one type of pneumococcus (Type II) react in about equal degree with all three types of antipneumococcus sera, and not with anti-typhoid or normal horse serum. The preparations of protein derived from Pneumococcus Type II by the method employed in the present investigation, therefore, seem to be species-specific rather than type-specific. This fact, if confirmed by subsequent investigation of the protein of pneumococci of other types, would indicate on the basis of specific precipitin reactions that all pneumococci possess in part a common basal specific protein. It must be borne in mind, however, that the immune sera used in these tests were not prepared by immunization with the isolated protein of the cell but by inoculation of horses with the intact bacteria. Immune sera are now being prepared from animals injected with purified protein as free as possible from other cell constituents such as the non-protein soluble substance. Until this is completed, it would, of course, be premature to attempt any interpretation of the significance of these reactions in terms of biologic specificity.

DISCUSSION.

It seems justifiable, then, on the grounds of the data presented in this and the preceding paper to conclude that the pneumococcus cell possesses at least two distinct substances which are intimately concerned with the biologic specificity of this organism. As a corollary of this, it would appear that pneumococcus immunity is related to two entirely distinct bacterial substances. One of these cellular constituents is the so called soluble specific substance, which as already pointed out is non-protein in nature and in its present state of purity is either carbohydrate or intimately associated with car-

bohydrate. This substance is chemically analogous to the bacterial gum which other investigators have isolated from various pathogenic and non-pathogenic encapsulated bacteria (3). However, previous studies have not related the chemical constitution of these polysaccharides to the serological or antigenic specificity of the organisms from which they were derived. The polysaccharide of pneumococcus, whether capsular substance or not, either is serologically reactive itself or is closely associated with some other substance which confers upon the organism the dominant character of type specificity. The protein fraction of the bacterial cell, on the other hand, is not type-specific but reacts in antipneumococcus serum regardless of type derivation. It is therefore species-specific, not type-specific.

The antigenic properties of these two substances; the relation of changes in specificity of pneumococcus to changes in virulence; and the relation of these substances to phenomena of infection and immunity are now being investigated.

SUMMARY.

1. The protein precipitated by acetic acid from solutions of pneumococci shows chemical reactions characteristic of nucleoprotein and mucoid.

2. The protein of pneumococcus, as contrasted with the non-protein soluble specific substance, exhibits species specificity rather than the type specificity characteristic of the latter.

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OBSERVATIONS ON THE HEALTH AND GROWTH OF CHILDREN IN AN INSTITUTION.

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(Received for publication, April 19, 1923.)

Through the courtesy of the trustees of a semicharitable institution for children of both sexes, we have had the opportunity to make observations for thirty-one months on the growth and nutrition of the children who were inmates. The data obtained have furnished some very interesting statistics on the growth and general condition of children living under institutional conditions.

THE INSTITUTION.

The institution was a Roman Catholic home and school for poor children, conducted by Sisters of a French order. During the first twenty months of our study, it was housed in an old-fashioned building occupying a plot about 100 feet square in the heart of the uptown business section of New York, which had been its home for over fifty years. This building was surrounded by tall office buildings, which cut off from it much light and fresh air. It was, therefore, dark and rather gloomy, though scrupulously clean. The refectories and kitchen were in the basement, requiring artificial light. The schoolrooms and dormitories were on the upper floors and had light on at least one side. The children were divided according to age into three groups for each sex. They slept in dormitories accommodating from twenty to thirty children each. Each child had a separate cot. The bathing facilities consisted of shower baths and wash bowls. The playgrounds, one for the boys and one for the girls, consisted of two small yards in the rear of the building, enclosed by high brick walls, the size of each being about 30 by 40 feet.

A summer home in the country was maintained where each child spent one month, some of the children going in July and the rest in August. A few who were in special need of the vacation were allowed to remain through the two months.

In January, 1922, the institution was moved to a country estate about 30 miles from New York. The new home consists of several buildings providing ample space and plenty of light and air. There are playrooms in the dormitory building and in the schoolhouse. There are two large outdoor playgrounds, one for each sex, equipped with swings, slides, etc.

INMATES.

Most of the children of this institution are not of the poorest classes, since as a rule a moderate fee is charged for their care. A few of the children were orphans; many were half-orphans, and some had both parents. The nationalities were chiefly French, Spanish, Italian and Irish. On the first examination and at subsequent observations, the children were with but few exceptions in excellent physical condition. They were happy and gay, not suffering in the least from institutional repression, although they were living under strict regulations. The schoolwork is the same as that of the public school. No systematic physical education is given, although the girls and small boys have lessons in folk dancing and dramatics.

The ages of the boys ranged from 4 to 14, the girls from 4 to 16. Many of the children remain in the institution for the entire period covered by the age limit. Some remain only a few months. There was, consequently, considerable change, from time to time, in the material on which our study was based.

GENERAL HEALTH.

The general health of the children is supervised by a visiting physician. He gives each applicant for admission a complete examination, and excludes defective children and those acutely ill or suffering from contagious diseases. Malnutrition is not a cause for exclusion, but is rather made a reason for admission. The rule of the institution is to quarantine for a least two weeks each child admitted. Minor ailments are treated by one of the sisters, who acts as nurse and administers

cathartics when necessary. About once in two weeks each child in the institution is given a dose of cascara sagrada as a precautionary measure. In the spring all children receive a tonic for a few weeks. Common colds are dealt with promptly by administration of a hot drink and an active cathartic.

The effectiveness of the general routine which is followed in the institution is manifest in the always excellent physical condition and the good digestion of the children. As an example, the cleanness of the tongues of the children was especially noteworthy. On one examination day special observations were made of the condition of the mouths. Of 144 children, only twenty showed even the slightest coating of the tongue, and only five showed marked coating, these being mostly older girls who were working in the kitchen.

During our study there was surprisingly little illness of any kind in the institution; no epidemics of any sort occurred, and almost no cases of serious illness. There were no deaths in the institution during the period of observation. One child was removed to a hospital suffering from appendicitis, from which he died. The infrequency of any important illness is further shown by the fact that in all the twenty-two weighing days, on each of which an average of 135 children were examined, there were only seventeen absences on account of illness.

When the institution was in New York many of the children had decayed teeth, fully 30 per cent. showing this condition at first examination. Since the removal to the country, much of this has been corrected. Adenoids and hypertrophied tonsils were found in about the usual frequency—nearly 30 per cent. of the children. During our period of study forty of the children had tonsillectomies. The children as a rule had excellent color, especially after removal to the country.

In general, it may be said that the surprisingly good physical condition of these children reflects great credit on the management of the institution. The impression is prevalent that children living in institutions decline in general health and vitality, and that their growth and physical development are inhibited. Our observations of these children show that this is not a necessary result, and that the contrary may be true. The carefully controlled life in a properly managed institution, the regular hours, the good food and the lessened chances of

infection, because the children do not come in contact with other children as do those who attend public school, make it possible to keep these children in excellent health, in fact, in much better condition than children in most private homes.

ROUTINE.

The children rise at 6 a.m., and wash and dress in silence. At 7 they have breakfast, which, like all meals is taken without talking. This practice has been followed because it was found that the children ate better than when talk and play were permitted. Like most children, they eat rapidly, seldom taking over twenty minutes for a meal, even a dinner of three courses. From 9 a.m. until 12 m., they are in school. At 12 m., dinner is served, and school follows from 1 to 3 p.m. From then until supper is play time. The older girls spend a portion of this time in sewing. The younger children have supper at 5 p.m. and go to bed at 6. The older children have supper at 7 p.m. and go to bed at 8. The older girls help in the general housework, and in the country the older boys assist about the grounds.

While in the city, the only outdoor exercise, except the time spent in the playyards, was a trip to Central Park on Sunday morning. The children walked to and from the park—about 1 mile—and played there for about one hour. In the country the children do considerable walking about the grounds, since the school is some distance from the other buildings. They play out doors each afternoon, the weather permitting.

From a hygienic point of view the conditions in the country are a great improvement over those in New York, but, as will be shown later, this change in environment apparently has had little effect either on health, weight gain, or general appearance of the children, possibly because the physical condition was so good while they were living in the city.

DIET.

Until about one month before our observations were begun the regular diet included little milk, although in other respects it was excellent. Tea and cocoa containing a very little milk had been given. Some additional milk had been used in the preparation of other food. A

large amount of meat had been provided, it being served once and sometimes twice a day, and meat soup was served twice a day. A month before our observations were begun the addition to the diet of a pint of milk daily for each child had been made. The amount of meat served was somewhat reduced and no meat or meat soup was served at supper.

TABLE 1.

Menu for a Week.

	Breakfast	Dinner	Supper
MONDAY	Bread and butter Cocoa	Meat soup Mutton stew Bread and cake	Milk soup Bread and jelly Fruit
TUESDAY	Oatmeal Bread and jelly Cocoa	Meat soup Roast beef, cabbage Bread, bananas	Baked beans Bread and butter Milk
WEDNESDAY	Cornflakes Milk and bread	Soup with carrots, onion Veal, tomato stew Bread and cake	Rice pudding Bread and jelly Milk
THURSDAY	Hominy Milk Bread and jelly	Meat soup Boiled beef Potatoes Bread and apples	Tapioca pudding Bread and butter Cocoa
FRIDAY	Oatmeal Bread Cocoa	Julienne soup Fish or egg Bread and bananas	Milk soup Baked beans Bread and jelly
SATURDAY	Bread and butter Cocoa	Meat soup Mutton Lettuce Bread and prunes	Bread pudding Apple sauce Bread and milk

Table 1 gives a typical weekly diet after these changes had been made. This dietary, with only minor variations, has been used throughout the period of our study.

The cocoa served at breakfast was made with milk without adding water. At supper the children either drank milk plain or took it in the form of milk soup, that is, hot milk with some seasoning and with

bread added. Little butter was used. Soups were made from meat stock with the addition of vegetables, and were served with large pieces of stale bread added. The dessert was usually fresh fruit or small sweetened rolls or cakes, which were often donated by hotels or restaurants. The children have always received throughout the year a fair amount of green vegetables; since the removal to the country, this has been somewhat increased. All the children received the same food, the quantity only being varied according to the age. Bread was allowed *ad libitum*, and the amounts consumed were astonishing. Each child usually ate at least two large pieces at each meal and many, especially the larger boys, took four or five slices. In addition to the amount taken at meals, a large piece was usually served to each child in the afternoon.

In October, 1920, the approximate amount of food taken for two consecutive days by each child then in the institution was ascertained. From the data obtained the caloric intake of each child, the percentage of distribution of the calories and the amount of fat, protein and carbohydrate were calculated. As a whole, the total caloric intake was high, except in the case of some of the older girls. It is suspected, however, that the amounts recorded for this group did not represent their total food intake, since they were working in the kitchen and refectories and had ample opportunity to obtain additional food.

The average distribution of the calories taken was: fat, 22 per cent.; carbohydrate, 61 per cent., and protein, 17 per cent. This distribution is somewhat different from what we have considered ideal: viz., fat, 35 per cent.; carbohydrates, 50 per cent., and protein, 15 per cent. The fat furnished too low a proportion of the total calories, and the carbohydrate too high a proportion. The supply of vitamin A was therefore probably not up to the average. The carbohydrate intake was very high. This was mainly due to the large amounts of bread consumed. The protein intake was higher than the average. The protein intake per kilogram was liberal. About 56 per cent. was animal protein and 44 per cent. vegetable protein, a somewhat lower proportion of animal protein than the optimum. However, the generous total protein intake undoubtedly compensated for this. The supply of mineral salts, especially calcium, was possibly low, since only 1 pint of milk was given.

Chart 1 shows the calories per kilogram taken by forty-six boys and eighty-three girls. The line shown on the chart is that previously estimated by us as a desirable average.[1]

It will be seen that the children up to the age of 10 years took considerably more than the theoretical requirement, while those over 10 often received less. This was largely due to the manner of serving the meals to the children. The food for the entire dining room was brought to the table in large containers, and a sister served it to the

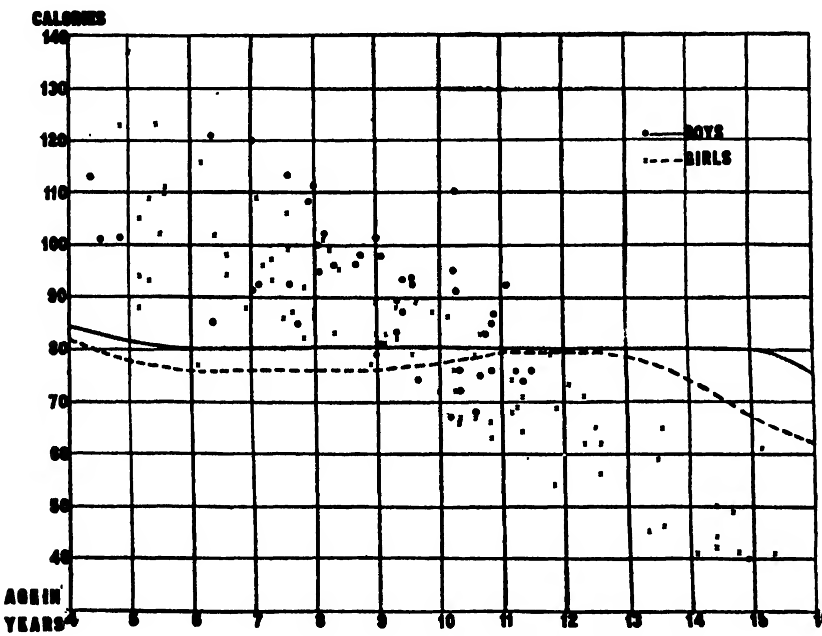

CHART 1.—Calories per kilogram taken by 129 children.

children, making some variation in amount according to the size of the child. By this practice, however, the smaller children received *proportionally* more than the larger ones. For example, a girl weighing 40 pounds (18 kg.) would receive at least half as much food as a girl weighing over 100 pounds (45.4 kg.). Of such items as fruit, cakes, etc., as well as milk, practically the same quantity was given to all the

1. Holt, L. E., and Fales, H. L.: Food Requirements of Children; Total Caloric Requirements, Am. J. Dis. Child. 21:1 (Jan.) 1921.

children, regardless of size. Therefore, since the amount of food taken was governed not by the desires of the children but by the judgment of the sister who served, the values are not to be taken as a guide for the

TABLE 2.

Typical Diet Sheet of a Medium Sized Boy (Average Amount of Food Taken for Two Days).*

Article of Food	Amount	Calories			
		Fat	Carbo-hydrate	Protein†	Total
Milk	16 oz.	149	94	69	312
Butter	1¼ tsp.	56	0	0	56
Cocoa	1 tsp.	7	4	2	13
Cake	0.5 piece	15	54	8	77
Gravy	2 oz.	11	3	2	16
Jelly	¼ oz.	0	25	0	25
Sugar	2 tsp.	0	49	0	49
Apple	50 gm.	2	22	1	25
Vegetables, mixed	1 tbsp.	1	5	1	7
Cabbage	4 oz.	1	2	4	7
Tomato	3 oz.	2	18	4	24
Meat	3 oz.	99	0	90	189
Soup	10 oz.	11	14	54	79
Rice pudding	4 oz.	62	141	35	238
Tapioca pudding	4 oz.	76	121	31	228
Bread‡	12 pieces	43	783	135	961
Total food		535	1,335	436	2,306
Calories per kilogram		23	57	18	98
Calories per pound		10	28	9	44
Distribution of calories		23	58	19	
Grams daily		58	326	106	
Grams per kilogram		2.4	13.8	4.5	
Grams per pound		1.1	6.3	2.0	

* W. A., aged 8 years and 9 months, weight, 52 pounds (23.6 kg.), height, 47 inches (117.5 cm.); average weight for height; undersize for age.

† Proportion of animal protein, 56 per cent.; of vegetable protein, 44 per cent.

‡ Amount includes that added to soup, tomato, etc.

normal caloric requirement. It is interesting, however, to note that the young children took eagerly so much food, and with apparently no ill effects.

Table 2 gives the form in which the diet of each individual was studied.

OBSERVATIONS ON GROWTH.

During the thirty-one months of our study, the children were weighed at frequent intervals, usually once a month, and the height was measured about once in three months. In all, 2,995 observations on weight were made on 346 children, 151 boys and 195 girls. The number of observations of each child varied according to the length of stay in the institution. Table 3 gives an idea of the number of observations for each child, and Table 4 the approximate duration of the period of observation of each child.

TABLE 3.

Number of Observations.

Number of observations	Boys	Girls	Total
18–22	21	41	62
12–17	18	18	36
7–11	37	54	91
4– 6	17	18	35
1– 3	58	64	122
Total	151	195	346

TABLE 4.

Duration of Observation.

Duration	Boys	Girls	Total
2 yrs.–2 yrs. 7 mos.	21	48	59
1½–2 yrs.	14	7	21
1 yr.–1½ yrs.	23	41	64
6 mos.–1 yr.	29	30	59
3–5 mos.	12	15	27
Under 3 mos.	30	43	73

The height was measured without shoes. The weights were taken without shoes, and in the usual indoor clothing. Heavy jackets or sweaters were removed. The children did not wear an institutional uniform, but there was much more uniformity in clothing than would

be found in a public school group. The clothing was simple, not excessive in weight, and varied very little for children of the same age and size. Various weighings were made of the average clothing worn in the winter and summer, not including shoes, coats and sweaters. This weight of the clothing was practically the same for boys and girls. It was found that for the large children, that is, those over 60 pounds (27.2 kg.) in weight and over 50 inches (125 cm.) in height, the average weight of winter clothing was 2 pounds; that of the summer clothing, 1.5 pounds. For the smaller children, weighing from 40 to 60 pounds (18 to 27.2 kg.) and ranging from 35 to 50 inches (88 to 125 cm.) in height, the average was 1.5 pounds for winter clothing and 1 pound for summer clothing. The weights taken have all been reduced to net

TABLE 5.

Distribution of Children According to Nationality.

Nationality	Number of Boys	Number of Girls	Total
American	9	14	23
French	27	30	57
Italian	37	47	84
Spanish	30	32	62
Irish	23	39	62
Balkan	6	10	16
Central European	19	23	42
Total	151	195	346

weight by making the deduction for clothing just mentioned. The purpose of this was to secure a more accurate comparison of the weights taken at different seasons. This correction for clothing is, of course, not absolutely accurate for children of all ages. However, since the same reduction to net weight was made in the successive weighings of each child, the gain or loss in weight is exact.

The data obtained at the initial examination of each child have been analyzed to determine the relation of the weight to height, weight to age and height to age, and the variation of each of these relations according to nationality.

The racial distribution is shown in Table 5. The Central European group includes mostly Germans, a few Russians, Austrians, Scandi-

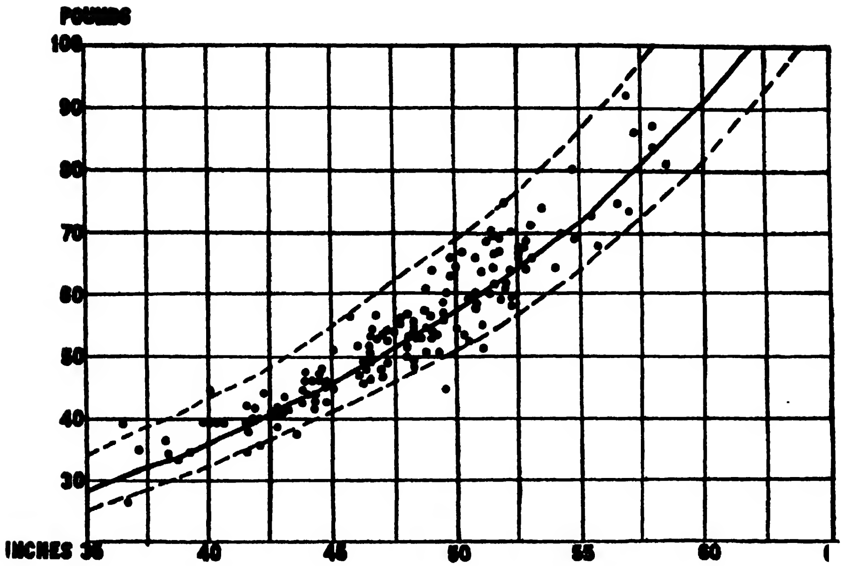

CHART 2.—Relation of weight and height (151 boys).

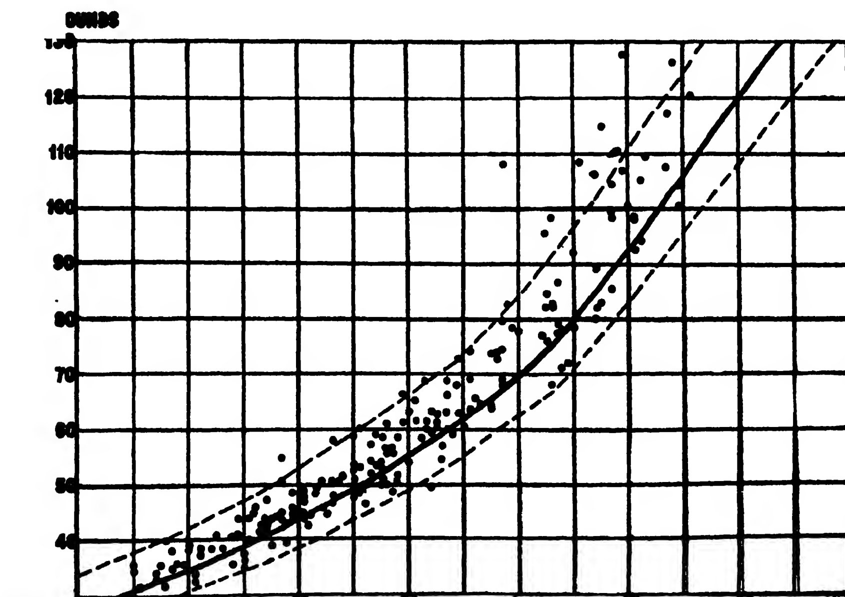

CHART 3.—Relation of weight and height (195 girls).

navians, Bohemians, one Hungarian and one Pole. The American group includes children born in this country and whose parents were also born here, but whose parentage was so mixed as to eliminate racial differences. Many of the children classed in the national groups were born in this country, but the parents were not; or the racial influence was so obvious that the classification was evident.

Relation of Weight to Height.—This relation on the first examination is shown in Charts 2 and 3. The solid line shows the standard weight for height and is based on the values given by Holt and Howland.[2] These values are derived from the figures obtained by ten different

TABLE 6.

Relation of Weight to Height in Different Nationalities.

Nationality	Normal Weight for Height (10% Below to 20% Above Average)		Under Weight for Height (More than 10% Below Average)		Overweight for Height (More than 20% Above Average)		Total Number of Children
	Number	Percentage of Total	Number	Percentage of Total	Number	Percentage of Total	
American	23	100	0	0	0	0	23
French	50	88	3	5	4	7	57
Italian	82	98	0	0	2	2	84
Spanish	56	90	2	4	4	6	62
Irish	58	94	0	0	4	6	62
Balkan	15	94	0	0	1	6	16
Central European	39	93	2	5	1	2	42
Total	323	93	7	2	16	5	346

authors and are based on more than 100,000 observations. The dotted lines in each chart mark the zone including the weights of boys and girls, respectively, 10 per cent. below and 20 per cent. above the standard weight for height. The charts show that the children of the institution were with very few exceptions near average weight for height, the tendency being slightly above the average.

A doubt has been expressed by some writers as to the propriety of using a single weight-for-height standard for different races. It was

2. Holt, L. E., and Howland, J.: Diseases of Infancy and Childhood, Ed. 8, New York, D. Appleton & Co., p. 23.

found that nationality had no apparent effect on the weight-height ratio of the children studied in the institution. Table 6 shows the exact relation of the height to weight and the influence of nationality on that ratio.

The adoption of a definite limit for the percentage below average weight as an evidence of malnutrition has frequently been questioned. It has been argued that, if this is done, some perfectly healthy children might be classed with those clearly not normal, especially if any definite variation according to race does exist. The most commonly accepted limits used in classifying children as normal in weight for height are from 10 per cent. below the average to 20 per cent. above. It is interesting to apply this standard to the group of children studied by us. There were only six boys and one girl who were more than 10 per cent. below average weight for height. Since there were so few underweight children, the details regarding them may be of interest. It will be noted that only one of these children had been in the institution more than one month when examined.

CASE 1.—Boy, French, aged 7 years and 4 months, examined six days after admission, and nutrition graded as II, had no physical defects. He remained in the institution only twelve days.

CASE 2.—Boy, French, aged 7 years and 4 months, examined fourteen days after admission, and nutrition graded III, suffered from rachitis of long standing, enlarged glands, bad teeth and scaly skin. Only one observation was made.

CASE 3.—Boy, Spanish, aged 4 years and 9 months, examined two weeks after admission, was mentally defective. He gained 3 pounds in the twelve months following the first examination.

CASE 4.—Boy, Spanish, aged 7 years and 7 months, examined one month after admission, and nutrition graded II, was highly nervous, with enlarged adenoids and tonsils (no operation was performed). He gained 5½ pounds in the next six months.

CASE 5.—Boy, German, aged 5 years and 3 months, examined two weeks after admission, and nutrition graded II, was one of twins (see Case 6). He had rickets of long standing, bad teeth and frequent minor illnesses. Inheritance was bad. He gained 5 pounds in weight and three-fourth inch in height in fourteen months.

CASE 6.—Boy, German, aged 5 years and 3 months, one of twins (Case 5), presented the same clinical picture as did his brother. He weighed a little more than the brother on admission, but made less progress. He gained 2 pounds in weight and 1.75 inches in height in fourteen months.

CASE 7.—Girl, French, aged 8 years and 6 months, examined two months after admission, and nutrition graded II, had bad teeth. She gained 1 pound in three months.

This proportion of underweight children is much smaller than that reported in most groups of schoolchildren. Whether the ample food and the excellent hygiene of the institution account for this is an interesting question. All but seventy-two of the children when first examined had been resident for at least one month and all but four for at least two weeks. It is probable that any previously existing underweight may have been corrected by the excellent food and hygiene even in as short a time as one month. It is the usual experience that children who are much below weight make a rapid initial improvement when they are given a proper diet and are placed in better environment. In selecting children for admission, preference was given by the admitting physician to those obviously in need of improved living conditions. In consequence, it is fair to assume that there had been some improvement in weight during the first few weeks residence.

Sixteen children were considerably overweight; that is, they were more than 20 per cent. above the average weight for their height at first examination. Nine of these sixteen overweight children were girls between 11 and 15 years of age, years in which a marked degree of plumpness is frequent. Of the sixteen children, four gained more than the average in the succeeding months; five gained at the average rate; two gained less than average, and one lost weight. Four did not remain in the institution long enough for subsequent observation. It is evident, however, that the condition of overweight had no definite relation to their further progress in growth.

Relation of Height to Age.—Wide variation of height in relation to age is seen in healthy children of all races. While the relation of height to exact age has little significance in individuals, it is interesting when groups are considered. Charts 4 and 5 show the relation of height to age. The children as a group, especially the girls, were short, as compared to the averages established from statistics obtained by the examination of mixed groups of American schoolchildren. Of 149 boys, sixty-four (43 per cent.) were about average height for age, or more than average height, and eight-five (57 per cent.) were not up to the average height for age. Of 195 girls, sixty-five (33 per cent.) were about average height for age, while 130 (67 per cent.) were under average height for age.

Table 7 gives the distribution of the values according to nationality. The Spanish children were the nearest to the average in height-age relation. The Italian children were shortest. The Irish as a group

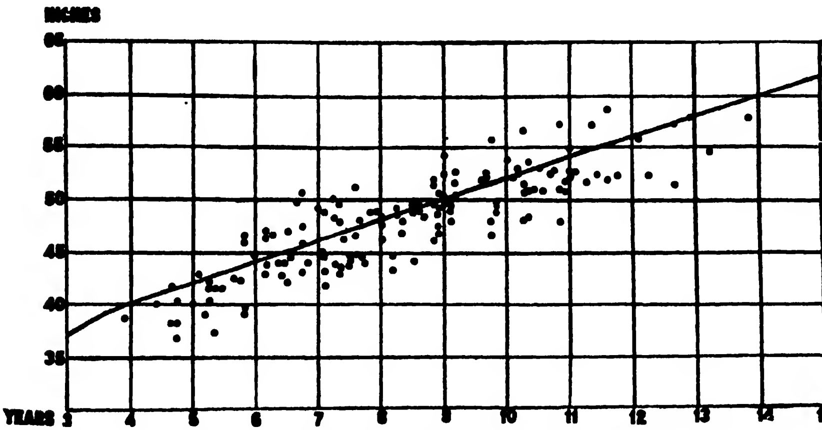

CHART 4.—Relation of height and age (149 boys).

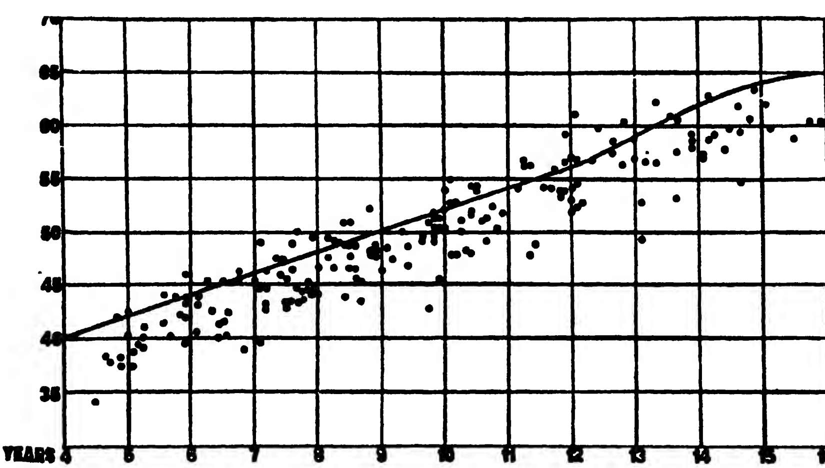

CHART 5.—Relation of height and age (195 girls).

were also short. It was of interest in studying the height to consider separately the children who had been resident in the institution for a long period before our observations were begun, since the diet pre-

viously had been notably deficient in calcium because of the small amount of milk given. Since the calcium supply is of undoubted importance in growth in height, it is not surprising that these children were short. Of eighty-nine children who had been resident in the institution for more than one year previous to our investigation, only eighteen (20 per cent.) were of average height for age, while seventy-one (80 per cent.) were under average height. Of these children, twenty-eight were as much as 4 inches (10 cm.) (almost two years' growth) under the average height for age, while ten were over 6 inches (15 cm.) under average height.

TABLE 7.

Relation of Height to Age in Different Nationalities.

Nationality	Above or About Average Height for Age		Under Average Height for Age		Total Number of Children
	Number	Percentage of Total	Number	Percentage of Total	
American	11	48	12	52	23
French	23	40	34	60	57
Italian	22	26	62	74	84
Spanish	33	53	29	47	62
Irish	20	33	41	67	61
Balkan	3	19	13	81	16
Central European	17	41	24	59	41
Total	129	37	215	63	344

Relation of Weight to Age.—The relation of weight to age showed, as would be expected, very wide variation, but on the average the children were near the usual weight for age. This was due to the fact that, although short, most of these children were of more than average weight for height.

Progress.—Most of the children who were under observation for a long enough period to allow any conclusions in regard to their progress in growth gained well and steadily in both weight and height. A critical survey of the rate of growth, however, showed that many of these children failed to make normal progress.

Table 8 shows the rate of growth of 148 children who were under observation for one year or more. The greater number of the boys

gained weight normally. Two thirds of them made also a normal gain in height. A little more than half the girls gained weight normally, and only one half made an average gain in height. When it is recalled that many of the children, especially the girls, were overweight or of average weight for age, these figures for rate of gain in weight are what might have been expected.

The progress in height is, however, a different matter. Most of these children were under average height for age, and it might have been expected, therefore, that in their good environment and with their liberal food allowance the height gain would proceed at more than average rate. There may be several reasons why this did not occur. It is possible that the diet, which included only 1 pint of milk daily,

TABLE 8.

Progress in Weight and Height.

	Total Number	Weight				Height			
		Progress Normal or Better than Normal		Progress Less than Normal		Progress Normal or Better than Normal		Progress Less than Normal	
		Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
Boys..............	55	44	80	11	20	36	65	19	35
Girls..............	93	54	58	39	42	48	51	45	49
All..............	148	98	66	50	34	84	57	64	43

was not adequate in mineral salts, especially in calcium. The observations of some recent writers, notably Sherman,[3] indicate that, since the other articles of food contain but little calcium, 1 quart of milk daily is needed by the growing child to supply a sufficient amount. Again, it is one of the observed facts in regard to growth that short children grow more slowly and tall children more rapidly than the average; so that, as a rule, short children become short adults, and tall children, tall adults. Many of these children belonged to short races, the Italian and French; but the short stature and slow growth were found in the case of the Irish, who are not racially short. Whether

3. Sherman, H. C., and Howley, E.: J. Biol. Chem. 53:375 (Aug.) 1922; J. Home Econ. 14:413 (Sept.) 1922.

it is possible to stimulate an established slow rate of growth by special diet is an interesting question. It has been proved possible within certain limits to do this with animals whose growth has been retarded by insufficient mineral salts, provided the retardation has not persisted too long.

TABLE 9.

Average Yearly Increment in Weight and Height.

Year of Age	Boys					Girls				
	Number of Cases	Weight Gain (Pounds)		Height Gain (Inches)		Number of Cases	Weight Gain (Pounds)		Height Gain (Inches)	
		Average	Normal*	Average	Normal*		Average	Normal*	Average	Normal*
4 - 4.5	1	1.0	2.0	1.25	1.25	0	...	2.0	....	1.25
4.5- 5	1	3.0	2.0	1.25	1.25	1	3.0	2.0	0	1.25
5- 5.5	3	1.5	2.0	1.25	1.00	6	1.3	2.0	1.0	1.00
5.5- 6	6	2.1	2.0	1.00	1.00	9	1.9	2.0	1.1	1.00
6 - 6.5	4	3.1	2.0	1.10	1.00	11	1.9	2.0	1.0	1.00
6.5- 7	7	2.9	2.0	1.10	1.00	15	1.9	2.0	0.75	1.00
7 - 7.5	8	2.0	2.4	1.00	1.00	14	2.6	2.25	1.00	1.00
7.5- 8	11	3.0	2.4	1.00	1.00	18	2.1	2.25	0.95	1.00
8 - 8.5	11	2.8	2.6	0.75	1.00	21	2.0	2.5	0.90	0.80
8.5- 9	13	2.9	2.6	1.00	1.00	20	2.9	2.5	1.0	0.90
9 - 9.5	20	2.2	3.0	1.00	1.00	21	1.6	2.6	0.95	1.10
9.5-10	14	2.9	3.0	1.00	1.00	13	2.8	2.6	1.1	1.10
10 -10.5	13	2.4	2.5	0.90	0.8	19	3.4	3.2	1.0	1.00
10.5-11	16	3.4	2.5	1.00	0.9	18	2.2	3.3	1.0	1.00
11 -11.5	11	3.8	3.2	1.10	0.9	12	4.8	4.7	1.2	1.25
11.5-12	6	3.9	3.3	1.00	0.9	9	2.3	4.8	1.15	1.25
12 -12.5	4	6.0	4.0	1.35	1.0	8	5.4	5.25	1.15	1.00
12.5-13	..	...	...	....	....	9	6.5	5.25	0.90	1.00
13 -13.5	..	...	...	....	....	8	2.5	4.8	0.95	1.00
13.5-14	..	...	...	....	....	6	7.1	4.7	1.0	1.00
14 -14.5	..	...	...	....	....	7	3.0	3.8	0.95	0.60
14.5-15	..	...	...	....	....	9	5.3	3.7	0.63	0.60
15 -15.5	..	...	...	....	....	5	3.3	3.0	0.45	0.40
15.5-16	..	...	...	....	....	1	2.5	3.0	0.50	0.30

* Holt and Howland: Diseases of Infancy and Childhood, p. 22.

Table 9 shows the average six months' increment in weight and height, according to age, of the children studied. For comparison, there is given the average six months' increment (Holt and Howland, p. 22), which has been derived from large numbers of observations. The standard values for six months are not absolutely exact, since

they have been obtained by dividing the usual yearly increment by 2, to obtain a value for a six months' period. The difference is unimportant. It is evident that the six months' growth of these children was, on the average, very near the normal. The fact that the average height gain was so near the normal is especially interesting in view of the observation previously made that many of the children gained in height at less than the average rate. It is evident that the others gained at enough more than the usual rate to compensate for those whose gain was less, thereby making the average very close to the values usually found.

Seasonal Growth.—A good deal of interest has been shown recently with regard to the variation in growth in different seasons of the year. The figures published by Porter[4] indicate that there are such variations, and that these are quite regular; viz., that the increase in weight during the six months from June to December far exceeds that in the six months from December to June. Gebhart[5] has recently published figures showing exactly what percentage of the annual gain in weight may be allocated to each of the calendar months. In his theoretical schedule, 60 per cent. of the gain for the entire year is made in the four months August to November, inclusive. The observations analyzed by him were quite numerous, but since his conclusions were based, partly upon the growth of one group of children who were followed for only four consecutive months and partly upon the growth of other groups who were followed but eight months, they seem hardly warranted by his facts.

Seasonal variations in growth have been ascribed to changes in environment, in activity and in diet, and to minor illnesses. It was thought that observations on the children studied by us might furnish some interesting data on this point, because their life was so nearly the same for the entire year. Their diet was known both qualitatively and quantitatively, and seasonal changes in diet were slight. Except for a single month in summer, their activity varied but little during the different periods of the year. Their environment remained the same.

4. Porter, W. T.: Am. J. Physiol. 52:121 (May) 1920.

5. Gebhart: Height and Weight as an Index of Nutrition, Bull. New York Ass'n for Improving Condition of the Poor, 1922.

Besides, as a group they were remarkably free from serious or even minor illnesses.

We have analyzed the weight records with reference to the following points: (1) a comparison of the winter and spring period (December to June) with the summer and autumn period (June to December); (2) the average gains made in each calendar month by boys and girls, and (3) the average gains for each successive month of the children who were followed for long periods. The average gain of the girls in 187 summer periods was 3.26 pounds; and in 144 winter periods, 3.34 pounds. The average gain of the boys in 113 summer periods was 3.50 pounds, and in eighty-five winter periods, 2.44 pounds. In the girls, there was therefore little difference between the summer and winter periods, the winter period showing, however, a slight increase. In the boys, the average summer gain was greater by one pound.

In graphic Chart 6 are shown the average gains by calendar months for boys and girls. Not all of the children were observed throughout the entire period, some being studied for two and a half years, some for only one year and some for only a few months. There were not less than forty-three and not more than fifty-five boys weighed each time, and not less than sixty-eight and not more than ninety girls. In 1920, there was a period of four months, June to September; in 1921, one of three months, July to September, and in 1922, one of two months, August and September, in which monthly weighing could not be made. The total weight gain for these periods has, therefore, been divided equally into monthly increments. Accordingly, the values for these months, as shown on the charts, are somewhat arbitrary.

There was much less variation shown in the gains of the girls than in those of the boys. The loss in weight in May and June, 1922, for the boys, and in June and July of the same year for the girls may be, in part, explained by more than usual activity, since this was their first warm season in the country, removal from the city having occurred in the previous January. This explanation is not entirely satisfactory, as the girls made good gains in May, the month in which the greatest loss for the boys is recorded.

About the only conclusion that can be drawn from these charts is that monthly gains are not at all regular even in healthy children with the same food, the same habits, and the same environment, and that

periods of two or three months of little gain are likely to be followed by a period of great gain, as is strikingly shown by the boys in 1921. The difference in gain during the same calendar months of successive years is also striking. June, November and December, especially, show wide variations in different years for both sexes.

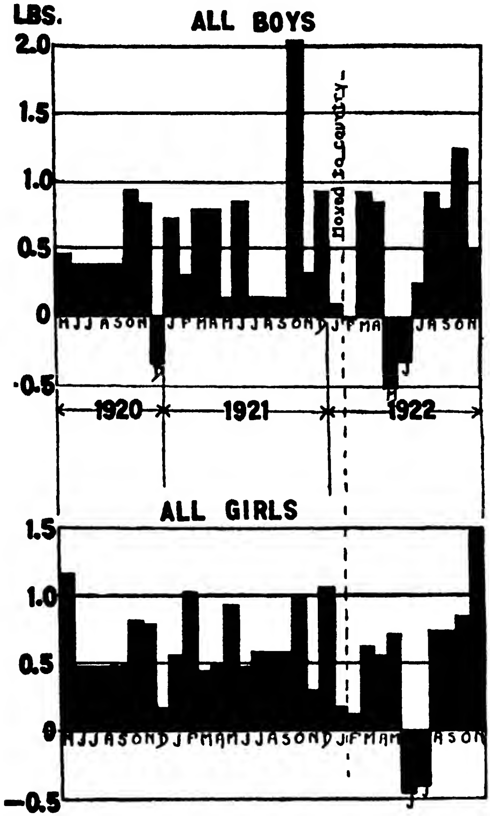

CHART 6.—Average gain in weight by months.

The great variation in monthly gains is even better brought out in Chart 7, where are shown the average gains for the children who were observed continuously during practically the entire period of our study. Although this is not a large group, the results are very striking. In considering these observations, the fact that we have grouped together children of different ages does not vitiate any conclusions, since the same children were observed throughout the entire period.

Chart 8 gives the average increment in weight for each calendar month for fifty-eight boys and for 116 girls who were under observation continuously for one year.

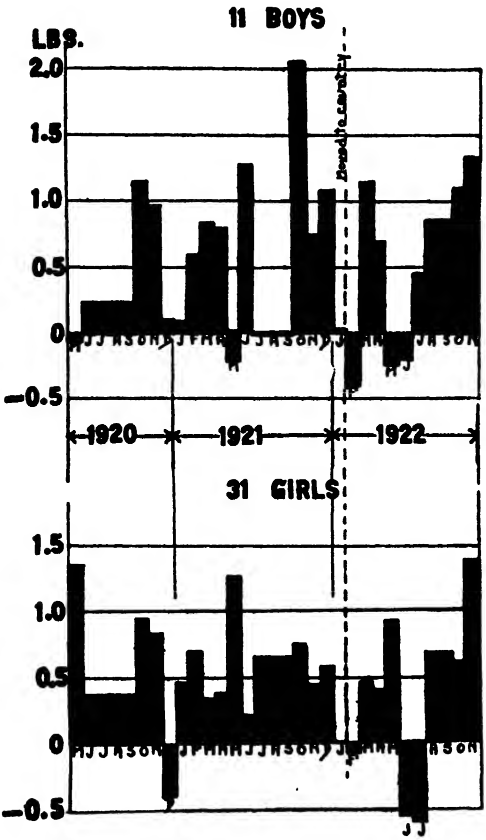

CHART 7.

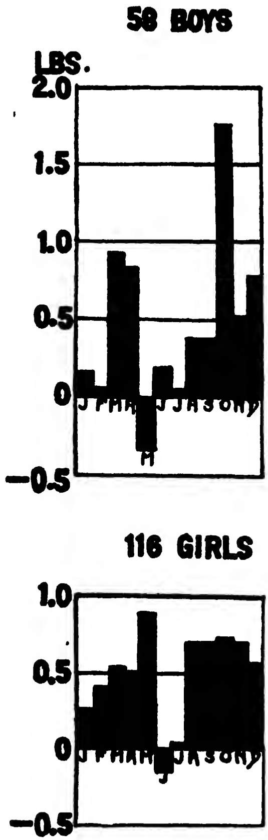

CHART 8.

CHART 7.—Average monthly weight gain in children observed for thirty-one months.

CHART 8.—Average monthly weight gain in children observed for one year.

It seems quite clear that it cannot be laid down as a rule that a healthy child gains a certain amount each month, nor that at any particular month or period the increase in weight is regularly more rapid, and at another period regularly slower, than the average rate for the year. The tendency, however, is certainly toward a more rapid gain in

the autumn months. The fact that the children studied by us, in regard to whom seasonal differences in diet and environment were insignificant, showed a marked, but irregular variation in monthly gain, would seem to indicate that the seasonal variation found by others is not due chiefly to the factors mentioned. It seems probable that a difference in activity may account for some of the difference between the summer and autumn gains, since the marked activity of the vacation months is usually reduced when the children spend four or five hours a day in school.

The one thing which was found to be fairly regular in these children was the annual increment. This was quite uniform. In this respect, our observations are in close accord with those of others. The regularity of the annual increment is of great significance in establishing a standard for healthy growth.

It seems of interest to append a few individual growth charts which are typical of certain groups of the children studied.

Chart 9 shows the growth curves for two years of a girl, 10 years old at the beginning of observation, who was of average weight for height and average size for age. The net progress in both weight and height in the two years proceeded at the normal rate, although there were marked fluctuations in weight, there being several periods of four or five months in which no net gain occurred. It will be noted that these periods of slow gain had no regular relation to season.

Chart 10 shows the growth curves for two and a half years of a girl, 8 years and 6 months old at the beginning of observation, who was at that time of average weight for height and average size for age, but who grew at better than the average rate. The fluctuations in the weight gain are especially marked. There are two periods in which there was continuous weight loss for about five months, followed, however, by a rapid gain in weight. This case illustrates especially the possible fallacy in judging the condition of the child and the progress in nutrition by following the weight for a short time.

Chart 11 shows the growth of a boy, 9 years and 6 months old at the beginning of observation, followed for two years, who was of average weight for height and a little more than average size for age, and whose growth proceeded at about the usual rate. Especially noteworthy is the marked drop in weight without evident cause in June and July of the second year, followed by a rapid increase which more than compensated for the decline.

Chart 12 shows the growth of a boy, 11 years old at the beginning of observation, followed for over two years. He was undersize for his age at the beginning of observation, and grew at considerably more than the average rate. The progress in this case was regular. This case, like the others presented, illustrates especially the fact that gains in weight and height usually proceed together.

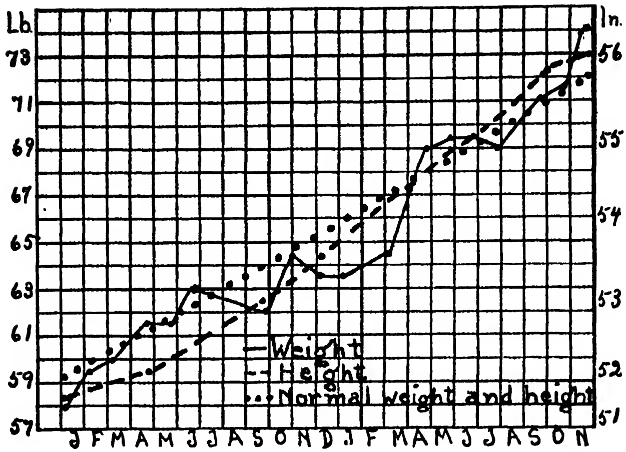

Chart 9.—Gain in weight and height of A. P., girl, French (initial age, 9 years and 11 months).

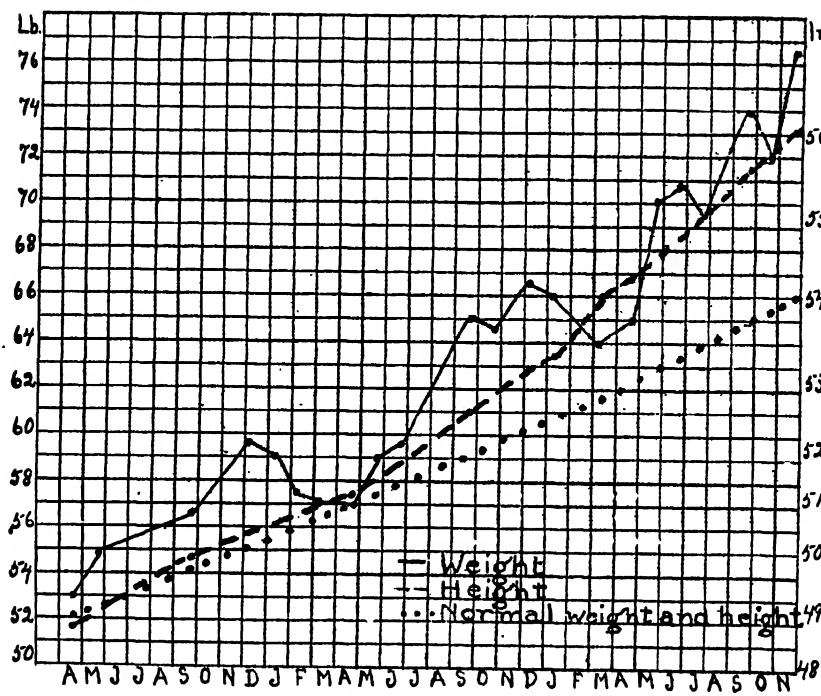

Chart 10.—Gain in weight and height of E. M., girl, Austrian (initial age, 8 years and 5 months).

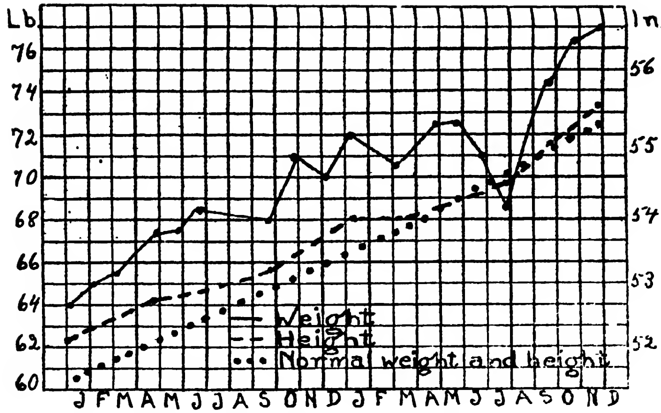

CHART 11.—Gain in weight and height of M. C., boy, French (initial age, 9 years and 8 months).

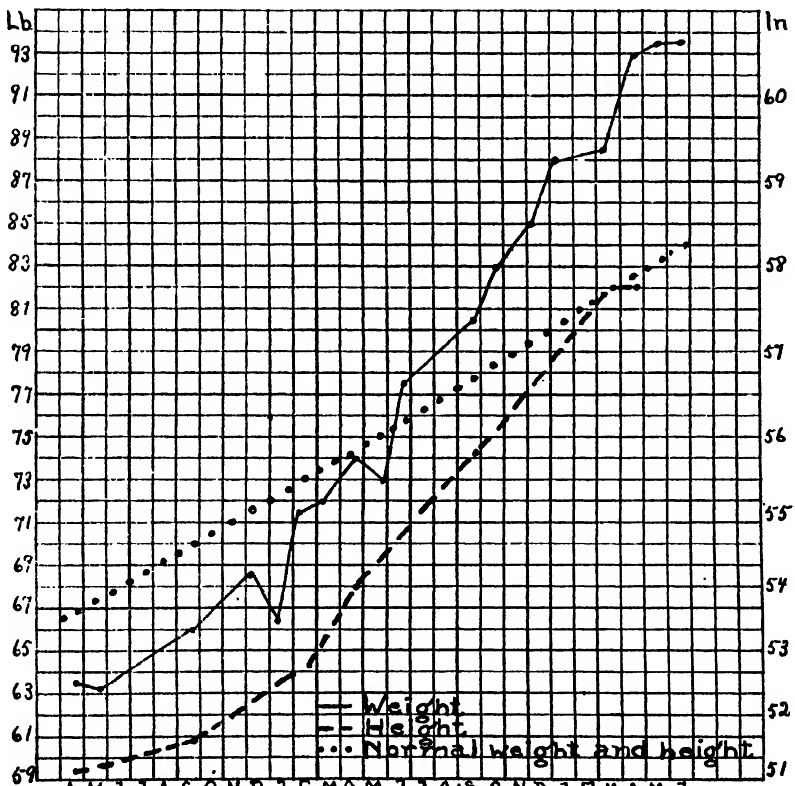

CHART 12.—Gain in weight and height of D. C., boy, Italian (initial age, 10 years and 10 months).

SUMMARY.

1. The observations show that strikingly good health and excellent nutrition can be maintained in an institution. The factors which contributed to this result were: (1) regularity in habits of eating and in sleep, rest and exercise; (2) a liberal but simple diet, and (3) freedom from contact infections. This shows what might be the case in private families provided the same rules of simple and regular living were observed.

2. In the distribution of fat, protein and carbohydrate in the diet, there existed a considerable deviation from what is considered optimum. In spite of this, health and digestion remained excellent, and the gains in weight were satisfactory. This shows how wide a deviation is consistent with health, provided certain essentials in the diet are furnished, and that regularity in habits is maintained.

3. Growth possibly would have been better if more milk had been allowed, furnishing more fat, more vitamin A and more calcium.

4. The usually accepted standard for normal nutrition, that is, inclusion in this group of those who were not more than 10 per cent. below the average weight for height, was found to hold good when applied to these children in spite of the differences of nationality.

5. While these observations are not numerous, they were continued long enough to show very clearly that in this group there were no regular seasonal variations. This is especially significant since the living conditions were so nearly uniform throughout the year.

6. The annual rate of gain was remarkably regular, though the monthly gains showed wide fluctuations, with no regularity whatever. This emphasizes the necessity of prolonged observations before a stationary weight can be considered significant.

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STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN THE BLOOD.

V. FACTORS CONTROLLING THE ELECTROLYTE AND WATER DISTRIBUTION IN THE BLOOD.*

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CONTENTS.

* The results given in this paper were presented at the meeting of December 4, 1922, to the Peking section of the Society for Experimental Biology and Medicine, and an abstract is published in the Proceedings for that meeting.

THEORETICAL.

Introduction.

The present paper is devoted to the quantitative formulation and experimental trial of a physicochemical explanation for the distribution of electrolytes and water between cells and plasma, and of the influence on the distribution of such factors as addition or loss of carbon dioxide, oxygen, acids, alkali, and salt. Such an explanation would cover, among other things, the facts that chloride and bicarbonate anions, though freely diffusible through the cell membrane, may be only half as concentrated in the cells as in the serum, and that the cell contents are more acid than the serum, although the H^+ (or OH') ion also is freely diffusible. It would cover also the mechanisms by which the cell buffers, though not diffusible, exert their buffer effects in the serum, and by which the cells take water from the serum when the carbon dioxide tension is raised. It is furthermore probable that the same laws which govern water and electrolyte distribution between intracellular and extracellular fluids in the blood, also govern these distributions in the other parts of the body, and control the relationships between the tissue cells and extracellular fluids, such as lymph and transudates.

The information concerning the amounts of base bound by oxyhemoglobin and reduced hemoglobin published in the third (1) and fourth (2) papers of this series indicated the possibility of a theoretical solution of the problem based upon the physicochemical laws of solutions, and upon the known facts concerning the reaction of the blood and the nature of its diffusible and non-diffusible ions and molecules.

Résumé of Facts Concerning the Blood Electrolytes.

The facts concerning the blood which may be accepted on the basis of data already in the literature are the following:

1. The osmotic pressure of the fluid within the cells appears to equal that of the serum outside. The disc shape of the erythrocyte indicates the absence of internal pressure. The latter would force the cell to assume a globoid shape, as it tends to do in hypotonic solutions.

2. In both cells and serum the positive charges of the alkali cations are balanced in part by negatively charged, non-diffusible protein anions, and in part by diffusible anions, of which Cl' and HCO_3' constitute the greater part.

3. All the non-protein ions normally present in amounts contributing significantly to the total osmotic pressure are monovalent. These are K^+, Na^+, Cl', and HCO_3' · Mg^{++}, Ca^{++}, SO_4'', and HPO_4'' are present in relatively such small amounts that in an approximation of conditions controlling the total osmotic pressure they may be neglected.

In Kramer and Tisdall's (3) summary of the results of analyses of the inorganic constituents of human serum, out of a total of 0.2912 gram equivalent of basic and acid radicles per liter of serum, Na, K, Cl, and HCO_3 accounted for 0.2787, or 95.6 per cent of the total. Of the remainder, Ca accounted for 0.0050 equivalent, or 1.8 per cent of the total, Mg for 0.9 per cent, HPO_4 for 0.0010 equivalent, or 0.3 per cent, and SO_4 for 0.004 equivalent, or 1.4 per cent.

In the cells Kramer and Tisdall estimate amounts of K, HCO_3, and Cl, accounting for 0.2001 out of a total of 0.2061 equivalent, or for 97 per cent of the total.

The cell inorganic phosphates on the basis of older analyses were, by one of us in a previous review (4) and in the introduction to the first paper of this series (5), classified with the non-diffusible electrolytes and accredited with an important share in the total osmotic and buffer effect. Using methods designed to avoid decomposing organic phosphates, Zucker and Gutman (6) have recently found that the blood phosphates are present in such small amounts as to play a quantitatively insignificant part in the total buffer and osmotic effects of the blood, and that they are probably diffusible.

4. Of the cell and serum proteins, only hemoglobin exerts a significant part of the total osmotic pressure.

That the electrolyte molecules and ions constitute nearly all of the osmotically active substances present is shown by the correspondence between the lowering of the vapor tension observed (Neuhausen, 7) and the lowering attributable to the electrolytes present. The chief non-electrolyte crystalloids, glucose and urea, themselves diffuse through the cell membranes and therefore cannot influence the water distribution. They are furthermore present in relatively small amounts, about 5 and 3 millimols respectively out of a total osmolar concentration of 300.

Of the proteins it appears that hemoglobin is the only one that exerts more than 1 per cent of the total osmotic pressure in either cells or serum. Hüfner and Gansser (8) found that electrolyte-free ox and horse hemoglobin exert the osmotic pressures calculated on the assumption that 1 molecule of oxygen combines at atmospheric pressure with 1 molecule of hemoglobin, and we have based our calculations of the osmotic effect of hemoglobin on these results. It will be seen from the tables that hemoglobin is estimated to exert about 10 per cent of the total osmolar concentration of the cells.[1]

The serum proteins, according to Starling (9) exert 30 to 40 mm. of pressure, or less than 1 per cent of the total (estimated at $0.300 \times \frac{311}{273} \times 22.4 \times 760 =$ 5,800 mm.). Presumably the cell proteins other than hemoglobin exert still less pressure, because of their small amount. It appears therefore that in calculating the total osmotic effects of the blood, the serum proteins and the cell proteins other than hemoglobin may be neglected.

We have adopted the convenient term "osmolar" concentration, introduced by Warburg (10), to indicate the total concentration of osmotically active ions and molecules.

5. The cell membranes are permeable to water, carbon dioxide, to the inorganic anions, and to either H^+ or OH', or both.

In water solutions the same $[H^+]$ would result, whether the membrane is permeable to $[H^+]$, or $[OH']$, or to both. The concentration of either ion varies inversely as that of the other, according to the equation $[H^+] = \frac{K_w}{[OH']}$. Any factor which fixes $[H^+]$ therefore fixes $[OH']$, and *vice versa*, so that it is impossible, and likewise for our present purposes immaterial, to tell whether the membrane is permeable for $[H^+]$, $[OH']$, or both.

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[1] Adair, in a personal communication, states that measurements that he has made in L. J. Henderson's laboratory indicate in dilute solutions a much higher molecular weight for hemoglobin than that found by Hüfner and Gansser, but that in concentrations approaching those in the cells other forces augment the osmotic power of the hemoglobin to about that which corresponds to Hüfner and Gansser's measurements.

6. The cell membranes are impermeable to the proteins, ionized or not, and to K and Na (Gürber, 11; Doisy and Eaton, 12).

7. The physiological pH ranges of the cells and serum are on the alkaline side of the isoelectric points of the cell and serum proteins (Michaelis, 13). Consequently, in the body the blood proteins combine with alkalies, but not with acids in amounts significant for the purposes of this paper.

8. The amounts of alkali bound by the cell and serum proteins increase in approximately a linear manner with increasing pH over the physiological range. The rate of change in protein-bound alkali per unit change in pH is several times as great in the cell fluid as in the serum (2).

9. At physiological pH ranges reduced hemoglobin binds less alkali (0.5 to 0.7 equivalent less per molecule of hemoglobin) than does oxygenated hemoglobin (1, 2).

The Solution Laws Involved in Blood Relationships.

In combining the above facts to form an inclusive quantitative expression of the phenomena of electrolyte and water distribution we have assumed for the blood the validity of the following physico-chemical laws:

I. At and near the neutral point all strong alkalies in quantitatively significant amounts are in the form of salts. At blood reaction, therefore, the total base B may be represented as BP + BA, where BP represents the alkali protein salts, in *equivalents of monovalent alkali*, and BA the salts formed by the alkali with other negative radicles, chiefly Cl' and HCO_3'.

II. The law of Donnan governing the influence of non-permeating ions on the distribution of permeating ions on the two sides of a membrane holds for the membranes of the blood cells. Donnan's theory has been provided with a basis of experimental facts by Donnan (14), by Procter and Wilson (15), and, for protein solutions, especially by Loeb's (16) recent studies.

III. The osmotic activity of each solute is proportional to the ratio $\frac{n}{N}$, of gram molecules of solute to gram molecules of water.

The presence of the serum proteins, according to the vapor tension determinations of Neuhausen (7), does not affect the validity of this ratio as the governing factor of osmotic activity, and data to be presented in this paper show that the cell proteins likewise fail to affect it. With dilute water solutions it makes relatively little difference whether the ratio $\frac{n}{H_2O}$ or $\frac{n}{\text{volume}}$ is taken as a measure of osmotic activity. In the blood cells, however, where the water constitutes only 60 to 65 per cent of the total contents, the difference is of importance.

Bjerrum (quoted by Warburg (10)) considers the ratio $\frac{n}{N + n}$ to be a better indicator of osmotic activity in concentrated solutions than the ratio $\frac{n}{N}$. In blood, however, n is less than 0.01 as great as N, so that within the limits of experimental error it is immaterial which of these two ratios we use. We consequently shall employ the simpler, $\frac{n}{N}$.

For our calculations, in place of using gram molecules of water as the unit of the denominator, we have used kilos of water, in order to express the results in terms not unnecessarily removed from the familiar gram molecules per liter unit.

The relationships expressed above under I, II, and III, may be expressed in certain basic equations, which when combined yield a practicably simple expression indicating the quantitative relationships of the factors discussed.

I. For the approximate *neutrality* of the blood reaction, [OH′] and [H+] being negligible compared with the other ions, we have

$$[B]_s = [BA]_s + [BP]_s \tag{1}$$

$$[B]_c = [BA]_c + [BP]_c \tag{2}$$

The brackets are used to indicate concentrations in terms of the ratio $\frac{\text{solute}}{\text{water}}$. The subscripts $_s$ and $_c$ indicate serum and cells, respectively. B, BA, and BP have the significance used under I in the

preceding discussion. (For simplicity we indicate all the alkali bound to non-diffusible acids as BP, although a small part may be bound by substances other than proteins such as conjugated phosphates.)

II. For conformity with *Donnan's law* (14) the *diffusible* monovalent ions have the following relationships:[2]

$$(3) \qquad \frac{[H^+]_s}{[H^+]_c} = \frac{[Cl']_c}{[Cl']_s} = \frac{[HCO_3']_c}{[HCO_3']_s} = \frac{[OH']_c}{[OH']_s} = \frac{[A']_c}{[A']_s} = r$$

A'_c and A'_s represent the sums of all the monovalent anions. For convenience we shall use the factor r to express the ratio indicated.

$[B]_c$ and $[B]_s$ do not appear in Equation 3, for they are not diffusible. If they were, in addition to the conditions defined in Equations 1, 2, 3, and 4, we should be required to make our final equation conform to the condition that $\frac{[Na]_s}{[Na]_c} = \frac{[K]_s}{[K]_c} = r$, and the results would be altogether different.

The relationships expressed in Equation 3 have already been pointed out by Warburg (10) and by Barcroft, Bock, Hill, Parsons, Parsons, and Shoji (17).

Barcroft and his collaborators (17), however, apparently used as a measure of activity the $\frac{\text{molecules of solute}}{\text{volume of solute}}$ ratio which is valid for dilute solutions of substances of small molecular weight. Warburg in his consideration of the problem realized that the volume ratio is not a close measure of activity in solutions containing as great a bulk of protein as the cell contents, and multiplied by the factor $\frac{\alpha_{CO_2} \text{ in water}}{\alpha_{CO_2} \text{ in blood}}$ the H_2CO_3 concentration per unit volume, and also the Cl' and HCO'_3 concentrations.

In applying Donnan's law Barcroft and his collaborators have assumed that the amount of alkali bound by hemoglobin is negligible. "Of the total ionic charges inside the corpuscles hemoglobin can provide, owing to its enormous molecular weight, only a negligible part." Since the appearance of Barcroft and

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[2] For a divalent anion, such as SO_4'' or HPO_4'', the distribution factor, according to Donnan's law is indicated by

$$r = \frac{[Cl']_c}{[Cl']_s} = \frac{\sqrt{[SO_4'']_c}}{\sqrt{[SO_4'']_s}} = \frac{\sqrt{[HPO_4'']_c}}{\sqrt{[HPO_4'']_s}}$$

his collaborators' paper, the experiments of Van Slyke, Hastings, Heidelberger, and Neill (1) have demonstrated that because of the polyvalent acid character of the hemoglobin molecule it combines at pH 7.4 with an amount of alkali in the cell about equal to that in the form of chloride. Of equal importance is the fact that practically all the *changes* in the ionic content of the cell that occur with varying CO_2 and O_2 tensions originate in the variations which CO_2 and O_2 tensions cause in the amounts of alkali bound by hemoglobin.

Warburg developed formulas in which the buffer effects of (oxygenated) cell and plasma proteins were indicated, but in which the actual amounts of base balanced by negative protein charges were not introduced. The data for estimating these amounts were, of course, not available at the time of Warburg's publication.

III. For *osmotic equality* the ratio of osmotically active molecules and ions to water is the same in serum and cells.

$$(4) \qquad \frac{\Sigma M_s}{H_2O_s} = \frac{\Sigma M_c}{H_2O_c} \text{ or } \Sigma[M]_s = \Sigma[M]_c$$

In Equation 4, M_s and M_c represent the osmotically active ions and molecules, and Σ $[M]_s$ and Σ $[M]_c$ the sums of their total concentrations, in terms of the $\frac{\text{solute}}{\text{water}}$ ratio, in serum and cells, respectively.

As alternative forms of Equation 4, we may write, if we assume complete dissociation of the electrolytes:

$$(5) \qquad [B]_s + [Cl]_s + [HCO_3]_s = [B]_c + [Cl]_c + [HCO_3]_c + [Hb]_c$$

$$(6) \qquad 2\,[BA]_s + [BP]_s = 2\,[BA]_c + [BP]_c + [Hb]_c$$

$$(7) \qquad 2\,[B]_s - [BP]_s = 2\,[B]_c - [BP]_c + [Hb]_c$$

Equation 5 merely expresses the sum of the total ions in serum, and of ions plus hemoglobin molecules in the cells, complete dissociation being assumed, and likewise a balancing, in serum and cells respectively, of the small amount (not over 5 per cent of the total) of osmotically active substances (PO_4'', SO_4'', etc.) not represented in the equation. Hb is expressed in units of mols of O_2 capacity.

In Equation 6 the total osmolar concentration is represented as twice the molecular concentration of the salts with monovalent ions and cations (since each dissociates into two ions) plus once the

concentration of base in the form of protein salt, since the osmotic effect of BP is due to the alkali cation. In the cells we add also the osmotic effect of the hemoglobin, which is assumed to be the same regardless of the ionic charge of the hemoglobin molecules.

Equation 7 is derived from Equation 6 by substituting [B] − [BP] for [BA], according to Equations 1 and 2.

As stated above, Equations 5, 6, and 7 are theoretically accurate if the electrolytes are completely dissociated into osmotically active ions. The observed osmotic behavior of alkali salts in general does not justify the assumption that dissociation is complete, and Neuhausen and Marshall (18) from electrometric measurements have estimated that the sodium salts in blood serum are 83 per cent dissociated. However, if we assume, not complete dissociation, but *equal* dissociation of the salts in cells and serum, respectively, the relationships expressed in Equations 5, 6, and 7 still hold, not exactly, but so nearly that the deviations may be neglected for present purposes.

The theoretical inexactness of Equations 5, 6, and 7 when γ, the degree of dissociation, is less than 1, even though γ is equal on both sides of the membrane, arises as follows: When γ becomes less than 1, although [Cl], [HCO_3], and the part of [B] balanced by [Cl] and [HCO_3], are all multiplied on both sides of the equation by the same factor, $\frac{1+\gamma}{2}$, to give their osmotic activities, the part of B present as BP is multiplied by a smaller factor, γ, and the [Hb] by a larger factor, 1. The two deviating factors, γ and 1, however, are not greatly different from $\frac{1+\gamma}{2}$, which is their mean; they apply in blood to relatively small parts of the total osmotically active solutes; and they partially balance their effects, which, to judge from our experimental results, exceed but little if any our present limits of experimental measurement.

The basic assumptions made under I and II, and expressed in Equations 1, 2, and 3, stand on experimental data familiar in the literature. The assumption of equal $\frac{\text{solute}}{\text{water}}$ ratios in cells and serum, expressed under III in Equation 4, and in Equations 5, 6, and 7, is without experimental basis in the previous literature. It depends upon data in the present paper. These are summarized in Table I,

taken from the four experiments with horse blood described in the experimental section of the paper. The data given are those that fit into Equation 5, because these are all determined by direct analysis. Except in Blood 2, in which we believe the base determinations on the cells were low, the agreement is as close as the analyses could be expected to yield.

TABLE I.

Equality of Ratio $\frac{\textit{Mols Solute}}{\textit{Water}}$ *in Cells and Serum.*

Blood No.	pH_s	Serum. $B+Cl+HCO_3$	Cells. $B+Cl+HCO_3+Hb$
		mM. per kilo H_2O	*mM. per kilo* H_2O
1	7.66	289	272
	7.11	299	294
2	7.71	297	285
	7.42	307	292
	7.28	314	297
	7.11	318	299
3	7.75	286	285
	7.42	294	294
	7.08	303	303
4	7.69	290	289
	7.35	297	294
	7.06	302	303

Electrolyte Distribution.

Dividing Equation 6 through by 2 $[BA]_s$ and rearranging it, we obtain

$$(8) \qquad \frac{[BA]_c}{[BA]_s} = 1 - \frac{[BP]_c + [Hb]_c - [BP]_s}{2\,[BA]_s}$$

We may assume that, whatever the dissociations of the different salts with the monovalent anions, the salts with identical anions are dissociated to nearly the same extent in serum and cells so long as the concentrations do not differ greatly. This assumption appears justified even though the cations in the cell are nearly all K, while those in the serum are nearly all Na; for, whether conductivity or

freezing point data are considered, the differences in dissociation found between potassium and sodium salts with the same anions at similar concentrations are slight.[3] We may then write, with approximate accuracy

(9)
$$\frac{[BA]_c}{[BA]_s} = \frac{[A']_c}{[A']_s} = r$$

(From Equation 3, $\frac{[A']_c}{[A']_s} = r$.) From Equation 1, $[BA]_s = [B]_s - [BP]_s$. Substituting, in Equation 8, r for $\frac{[BA]_c}{[BA]_s}$ in the left hand member, and $[B]_s - [BP]_s$ for $[BA]_s$ in the right hand member, we obtain the following equation, showing *the approximate relationship between the distribution of diffusible ions and the amounts of alkali combined with the non-diffusible substances (proteins) of the cells and serum.*

(10)
$$r = \frac{[H^+]_s}{[H^+]_c} = \frac{[Cl]_c}{[Cl]_s} = \frac{[BHCO_3]_c}{[BHCO_3]_s} = 1 - \frac{[BP]_c + [Hb]_c - [BP]_s}{2\,([B]_s - [BP]_s)}$$

We may expect the three ratios in Equation 10 to vary from equality to each other, and to the r calculated from the right hand member of the equation, in proportion as the γ, and perhaps secondary factors affecting osmotic activity, vary in the cells from the like factors in the serum, but we may expect these variations in the ratios not to exceed a few per cent of their values.[4]

[3] See tables in Lewis (19), pp. 226–227.

[4] The relationship between electrolyte distribution and alkali bound by protein may also be derived as follows: From Equations 1 and 2, $[A]_c = [B]_c - [BP]_c$, and $[A]_s = [B]_s - [BP]_s$.

$$\text{Hence } r = \frac{[A]}{[A]_s} = \frac{[B]_c - [BP]_c}{[B]_s - [BP]_s}$$

This equation rests only on the assumption expressed in Equations 1, 2, and 3, and does not express or depend upon the equality of the $\frac{\text{solute}}{\text{water}}$ ratios expressed in Equation 4. It could not therefore be used for the further development of the theory into equations like Equations 14 and 23, which depend on the validity of the $\frac{\text{solute}}{\text{water}}$ ratio.

To obtain the value of r in terms indicating directly the effect of protein concentration, pH values, and degree of oxygenation, we substitute for $[BP]_s$ and $[BP]_c$ their values in these terms, as found in the experimental part of this paper and expressed in Equations 54 and 57. Making these substitutions in Equation 10 and combining the terms in which $[Hb]_c$ is a factor we obtain, as an approximation, accurate over a narrow pH range,

$$(11) \quad r = 1 - \frac{3.35\,[Hb]_c\,(pH_c - 6.44) - 0.068\,[P]_s\,(pH_s - 4.80) + [O_2]_c\,(0.25\,pH_c - 1.18)}{2\left([B]_s - 0.068\,[P]_s\left\{pH_s - 4.80\right\}\right)}$$

Equations 10 and 11 suffice for determining whether results obtained with a given blood agree with the quantitative requirements of the laws on which these equations are based. Because of the variation in water distribution with changing pH and oxygen content, however, the concentrations even of the non-diffusible constituents $[Hb]_c$, $[P]_s$, and $[B]_s$ are variable. Consequently, Equations 10 and 11 cannot be used to predict the r curve of a given blood with varying pH. However, by combining Equation 4 with 10, one is obtained in which all the values on the right side are functions of values which are constant for a given blood; *viz.*, $(B)_c$, $(B)_s$, (Hb), and $(P)_s$.

In the remainder of this paper we shall utilize parentheses to indicate units of substance *per unit of whole blood, e.g.* $(H_2O)_c$ = kilos of cell water per kilo of blood, $(P)_s$ = grams of serum protein per kilo of blood, and $(B)_s$ = millimols of serum base per kilo of blood, as contrasted with the bracketed $[B]_s$, which indicates the ratio $\frac{\text{serum base}}{\text{serum water}}$, or $\frac{(B)_s}{(H_2O)_s}$.

In Equation 10 we substitute $\frac{(BP)_s}{(H_2O)_s}$ for $[BP]_s$, $\frac{(Hb)_c}{(H_2O)_c}$ for $[Hb]_c$, etc. We thus obtain

$$(12) \qquad r = 1 - \frac{(H_2O)_s}{(H_2O)_c} \times \frac{(BP)_c + (Hb)}{2\left\{(B)_s - (BP)_s\right\}} + \frac{(BP)_s}{2\left\{(B)_s - (BP)_s\right\}}$$

From Equation 4 we have $\frac{(H_2O)_s}{(H_2O)_c} = \frac{\Sigma M_s}{\Sigma M_c}$. Substituting $\frac{2(B)_s - (BP)_s}{2(B)_c - (BP)_c + (Hb)_c}$ for $\frac{\Sigma M_s}{\Sigma M_c}$ (see discussion of Equation 7), we get

$$(13) \qquad \frac{(H_2O)_s}{(H_2O)_c} = \frac{2(B)_s - (BP)_s}{2(B)_c - (BP)_c + (Hb)}$$

Substituting in Equation 12 the value for $\frac{(H_2O)_s}{(H_2O)_c}$ from Equation 13, we obtain

$$(14) \qquad r = \frac{[H^+]_s}{[H^+]_c} = \frac{[Cl']_c}{[Cl']_s} = \frac{[HCO_3']_c}{[HCO_3']_s} = 1 - \frac{(BP)_c + (Hb)}{2(B)_c - (BP)_c + (Hb)} + \frac{(BP)_s}{2\left\{(B)_s - (BP)_s\right\}}$$

The effects of the pH and oxygen content may be introduced as in Equation 11 by substituting the values of $(BP)_c$ and $(BP)_s$ from Equations 54 and 57.

If the indiffusible substances, base and proteins, in the cells are assumed to maintain constant relations to each other, and the indiffusible substances within the serum are assumed to do likewise, it becomes possible to express as functions of (Hb) the other three constants, $(P)_s$, $(B)_s$, and $(B)_c$. Under these circumstances $(B)_c$ is proportional to (Hb), and the serum base and protein, $(B)_s$ and $(P)_s$, decrease by amounts proportional to (Hb). Thus, from the data in the experiments on normal horse blood reported in this paper, we have with a fairly close degree of constancy:

$$(15) \qquad (B)_c = 6.0\ (Hb)$$

$$(16) \qquad (B)_s = 148 - 8.3\ (Hb)$$

$$(17) \qquad (P)_s = 0.072 - 0.0039\ (Hb)$$

In Equations 16 and 17 the first numerical constant in each represents the average value at normal pH for serum free from cells, and when, therefore, (Hb) = 0. The second constants indicate the rates

of change in $(B)_s$ and $(P)_s$, respectively, per unit of increase in hemoglobin, when the hemoglobin is measured in terms of millimols of oxygen capacity per kilo of blood.

From inspection of Equation 14 it is evident that the fraction $\frac{(BP)_c + (Hb)}{2\,(B)_c - (BP)_c + (Hb)}$, expressing the effects of the cell factors, is at a given pH, constant for all bloods, whether of high or low hemoglobin content, as long as the ratio of base to hemoglobin in the cells remains constant. For then all the terms in both numerator and denominator vary directly as (Hb) (see Equations 15 and 55). The second fraction of Equation 14, $\frac{(BP)_s}{2\,\{(B)_s - (BP)_s\}}$, expressing the effect of the serum factors, varies slightly, at constant pH, with the hemoglobin content of the blood. But the variation is so small, and the total effect of this fraction on the value of r relatively so little, that the r value is, within the limits of experimental determination, independent of the hemoglobin content of the blood, even when the latter varies over such a wide range as from 3 to 12 millimolar, corresponding to from 7 to 27 cc. of oxygen capacity per 100 gm. of blood.

Consequently we may represent the average normal r, pH relationship by a single curve, which holds for bloods of varying hemoglobin content, if the other non-diffusible constituents maintain towards the hemoglobin the relationships indicated by Equations 15, 16, and 17. The curves obtained by substituting in Equation 14 the values for $(B)_c$ and $(B)_s$ indicated by Equations 15 and 16, (when Hb = 9) and the values for $(BP)_s$ calculated from the $(P)_s$ indicated by Equation 17, are given in Fig. 1. In plotting the curves the values of r were obtained by solving Equation 14 by a method of repeated approximation, for pH_c values of 6.4, 6.8, 7.2, 7.6, and 8.0. For the first approximation the serum factors were ignored, and r was estimated as $1 - \frac{(BP)_c + (Hb)}{2\,(B)_c - (BP)_c + (Hb)}$, $(BP)_c$ being estimated by interpolation on the curves of Fig. 10 (below pH_c 6.8 the curves are extrapolated beyond experimental data, and therefore are not so certain as above that point). From the preliminary r values thus obtained, a preliminary pH_s was estimated as $pH_s = pH_c + \log r$.

A close approximation of r was then obtained by using the whole of Equation 14, with this pH_s value for estimation of $(BP)_s$ by Equation 54 in the fraction $\frac{(BP)_s}{2\{(B)_s - (BP)_s\}}$. With the second value of r thus obtained the value of this fraction was once more estimated,

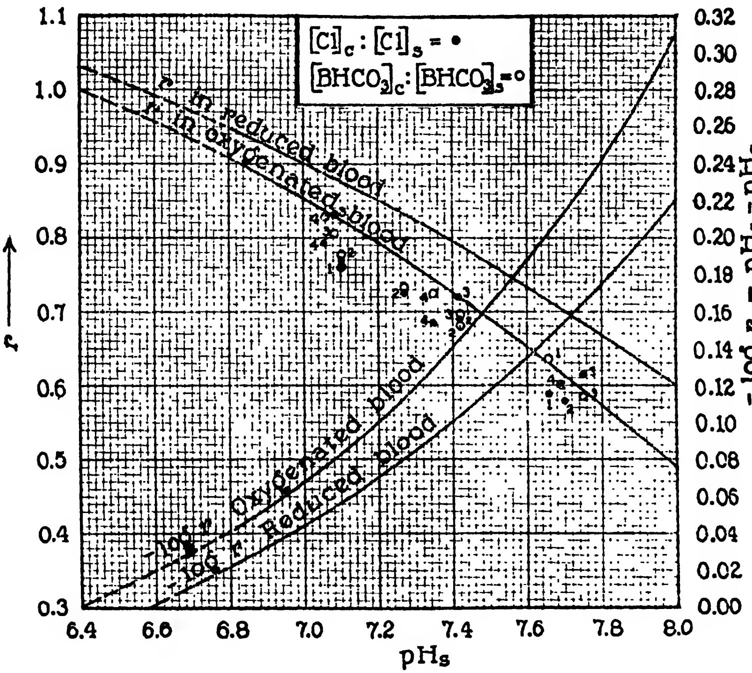

FIG. 1. Values of $r = \frac{[H^+]_c}{[H^+]_s} = \frac{[Cl']_c}{[Cl']_s} = \frac{[HCO_3']_c}{[HCO_3']_s}$ calculated by Equation 14 for horse blood of average serum and cell composition observed in the four experiments reported in this paper are indicated by the curves. The observed chloride and bicarbonate ratios in our experiments are indicated by the marked points.

and with it the final value of r, correct within a negligible limit of error.

In Fig. 1 the values for r obtained in the four experiments with oxygenated blood described later in this paper are indicated by solid circles for the $\frac{[Cl]_c}{[Cl]_s}$ ratio, by hollow circles for the $\frac{[BHCO_3]_c}{[BHCO_3]_s}$ ratio.

Their deviations from the mean calculated curve are partly due to the fact that the theoretically calculated r values tend to lie above the observed values, but more to the fact that the relationships of $(B)_c$, $(B)_s$, and $(P)_s$ to (Hb) vary in the individual bloods appreciably from the mean relationships indicated in Equations 15, 16, and 17, and used in plotting the curves of Fig. 1. In Fig. 12, where the individual $(B)_c$, $(B)_s$, and $(P)_s$, as well as (Hb), values found in each individual blood are used, the differences between the calculated and observed r values are smaller.

Since, according to Donnan's law as expressed in Equation 3, $r = \frac{[H^+]_s}{[H^+]_c}$, we may write

$$(18) \qquad -\log r = -\log [H^+]_s + \log [H^+]_c = pH_s - pH_c$$

The values of $-\log r$, therefore, indicate the pH differences between the serum and cells. These values we have plotted in the curves indicated in Fig. 1.

From data of quite a different nature, obtained on whole blood, serum, and hemolyzed blood and cells, and based in part on electrometric determinations, Warburg (10) has estimated the $pH_s - pH_c$ values in horse blood at varying pH_s. Comparison shows that our $-\log r$ curve is parallel throughout and nearly identical with the curve indicating the maximum $pH_s - pH_c$ values estimated by Warburg.[5]

Water Distribution and Cell Volume.

The distribution of water between cells and serum, and the resulting volume effects, may be predicted from the pH and the degree of oxygenation of the blood if the amounts of non-diffusible substance, *viz.* base and protein, in the cells and serum, respectively, are known, and if the law of equality of osmolar concentrations expressed in Equation 4 is valid for blood, as we believe it is demonstrated to be by experimental data in this paper.

[5] See Warburg (10), p. 230, Curve I, Fig. 11.

From the general statement expressed in Equation 4 we have

$$(19) \qquad \frac{(H_2O)_s}{(H_2O)_b} = \frac{\Sigma\,(M)_s}{\Sigma\,(M)_b} = \frac{\Sigma\,(M)_s}{\Sigma\,(M)_s + \Sigma\,(M)_c}$$

$$(20) \qquad \frac{(H_2O)_c}{(H_2O)_b} = \frac{\Sigma\,(M)_c}{\Sigma\,(M)_b} = \frac{\Sigma\,(M)_c}{\Sigma\,(M)_s + \Sigma\,(M)_c}$$

where $(H_2O)_s$, $(H_2O)_c$, and $(H_2O)_b$ represent the fractions of a kilo of water present, respectively, in the serum, cells, and whole of a kilo of blood; $\Sigma\ (M)_s$, $\Sigma\ (M)_c$, and $\Sigma\ (M)_b$, the total osmolar units (millimols) in the serum, cells, and whole of a kilo of blood.

Substituting for $\Sigma\ (M)_c$ and $\Sigma\ (M)_s$ their values as in Equation 7, and replacing $(B)_c + (B)_s$ by $(B)_b$ in the resulting equations, we obtain

$$(21) \qquad \frac{(H_2O)_s}{(H_2O)_b} = \frac{2\,(B)_s - (BP)_s}{2\,(B)_b - (BP)_s - (BP)_c + (Hb)}$$

$$(22) \qquad \frac{(H_2O)_c}{(H_2O)_b} = \frac{2\,(B)_c - (BP)_c + (Hb)}{2\,(B)_b - (BP)_s - (BP)_c + (Hb)}$$

Multiplying Equations 21 and 22 through by $(H_2O)_b$, we obtain

$$(23) \qquad (H_2O)_s = (H_2O)_b \times \frac{2\,(B)_s - (BP)_s}{2\,(B)_b - (BP)_s - (BP)_c + (Hb)}$$

$$(24) \qquad (H_2O)_c = (H_2O)_b \times \frac{2\,(B)_c - (BP)_c + (Hb)}{2\,(B)_b - (BP)_s - (BP)_c + (Hb)}$$

The above equations, the validity of which has been tested in the experimental part of this paper, enable one to predict the amounts of cell and serum water per unit weight of blood in terms which are either determinable constants $(B)_c$, $(B)_s$, and (Hb), for a given blood, or which may be calculated from such constants, *viz.* $(P)_s$ and (Hb), and from the pH and oxygen content. The effects of pH and of oxygen saturation may be introduced as in Equation 11.

Within limits the increase of volume produced by adding a solute to a solvent approximates a linear function of the amount of solute added, and in both cells and serum nearly all the variable solute is protein. We may, therefore, with approximate accuracy, write

$$\frac{V_c}{V_b} = G_b \left\{(H_2O)_c + m\ (Hb)\right\} \tag{25}$$

$$\frac{V_s}{V_b} = G_b \left\{(H_2O)_s + n\ (P)_s\right\} \tag{26}$$

where G_b is the specific gravity of the blood, with water at the same temperature as unity; m and n represent the volumes occupied in solution by a unit of cell and serum protein, respectively. When Hb and P_s are expressed in gram units, m and n both have values somewhat less than 1, since a gram of protein occupies somewhat less than 1 cc. volume. For horse blood we have found $m = 0.90$ and $n = 0.85$ when Hb and P_s are expressed as grams of protein. When Hb is expressed in millimols of O_2 capacity, $m = 0.90 \times 0.0167 = 0.015$.

The introduction of the factor G_b into Equations 25 and 26 is in accordance with the following considerations. If we assume that the blood proteins have the same coefficient of expansion as water, which assumption for the slight temperature effects involved can produce no significant error, then the volumes of cells and serum, respectively, per *kilo* of blood will be represented by the quantities $\frac{(H_2O)_c + m\ (Hb)}{D_{H_2O}}$ and $\frac{(H_2O)_s + n\ (P)_s}{D_{H_2O}}$, respectively ($D_{H_2O}$ = density of water at the temperature of measurement, that of water at 4° being unity). In order to change the volumes per kilo of blood to volumes per liter, we multiply the former by D_b, the density of blood, with water at 4° as unity. But $D_b = G_b \times D_{H_2O}$. Multiplying, therefore $\frac{(H_2O)_c + m\ (Hb)}{D_{H_2O}}$ and $\frac{(H_2O)_s + n\ (P)_s}{D_{H_2O}}$ by $G_b\ D_{H_2O}$, we obtain the volume values indicated in Equations 25 and 26.

Introducing the numerical values for m and n in Equations 25 and 26, we obtain

$$\frac{V_c}{V_b} = G_b \left\{(H_2O)_c + 0.015\ (Hb)\right\} \tag{27}$$

(Hb) being expressed in millimols of oxygen capacity per kilo of blood, and

$$\frac{V_s}{V_b} = G_b \left\{(H_2O)_s + 0.85\ (P)_s\right\} \tag{28}$$

$(P)_s$ being expressed as grams of serum protein per gram of blood (not, for this equation, as grams per kilo of blood).

The value of G_b and $(H_2O)_b$, constant for a given blood, may be estimated for normal horse blood as

$$G_b = 1.027 + 0.0037\ (Hb) \tag{29}$$

and

$$(H_2O)_b = 0.914 - 0.015\ (Hb) \tag{30}$$

The numerical constants in Equations 29 and 30 are obtained as described in connection with Equations 16 and 17, the first constant

TABLE II.

Calculated Effect of pH Change on Water Distribution Compared with Effect Observed by Warburg.

Blood constants estimated from hemoglobin content.

(Hb) = 11.3 observed.		$(B)_s$ = 54.2	from Equation 16.
$(P)_s$ = 0.0279	from Equation 17.	G_b = 1.069	" " 29.
$(B)_c$ = 67.8	" " 15.	$(H_2O)_b$ = 0.745	" " 30.

pH_s	$-\log r$ (from Fig. 1).	pH_c	$(BP)_s$	$(BP)_c$	$\frac{V_c}{V_b}$ Calculated by Equation 27.	Volume of cells in per cent of their volume at pH 6.8.	
						Calculated by Equation 27.	Observed by Warburg.
6.8	0.04	6.76	4.2	6.5	0.640	100.0	100.0
7.0	0.07	6.93	4.6	13.4	0.631	98.6	97.4
7.2	0.10	7.10	5.0	20.4	0.621	97.0	95.2
7.4	0.14	7.26	5.3	26.9	0.611	95.5	93.7
7.6	0.18	7.42	5.7	33.4	0.602	94.0	92.2
7.8	0.22	7.58	6.1	40.0	0.591	92.3	90.5

in each equation representing the G_b or (H_2O) value for normal serum, the second constant representing the change per unit increase in (Hb).

The agreement of the $(H_2O)_s$ and $(H_2O)_c$ values calculated at varying pH by Equations 23 and 24 with our observed values is indicated by the tables in the experimental part of this paper, and is summarized in Fig. 13.

Warburg (10) has estimated the changes in cell volume with varying pH by measuring the oxygen capacity of the cells. The number

of his determinations is sufficiently large to permit the plotting of an average curve by means of which the errors, that appear inherent in any method thus far used in estimating the small percentage changes in cell volume involved, are to a considerable extent neutralized. Warburg expresses his results in volume of cells at varying pH_s compared with the volume at pH_s 6.5. In Table II we have calculated for a blood, of the average hemoglobin content

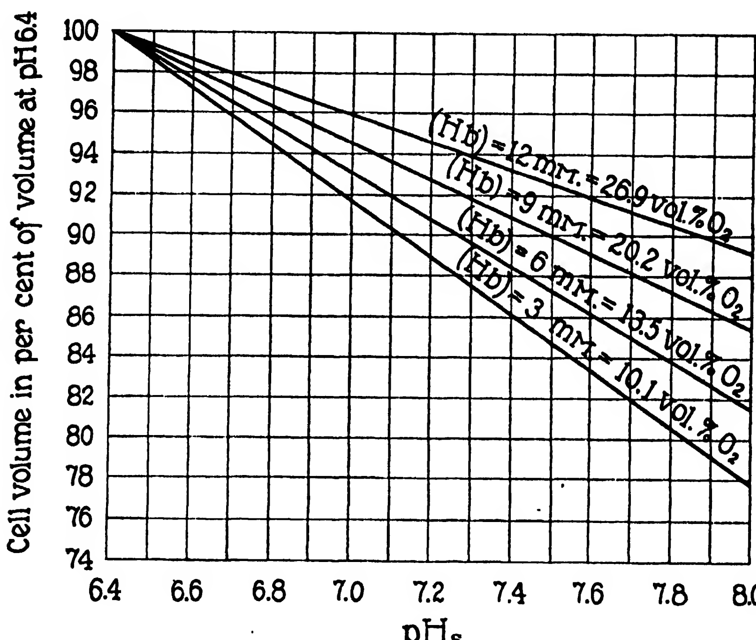

FIG. 2. Cell volumes calculated by Equations 24 and 27 for blood of average serum and cell composition observed in our experiments.

((Hb) = 11.3 millimolar) of the bloods used by Warburg, the change in cell volume as estimated by Equation 27. We have used as the unit of comparison the volume at pH_s 6.8 instead of pH_s 6.5, for the reason that both our experimental data and Warburg's are less complete and appear less certain below pH 6.8 than above it.

The changes observed by Warburg agree with those calculated by Equation 27 within the limit of experimental error, as do the changes observed by us, except in one experiment (No. 2). Warburg's ob-

served changes tend to exceed the calculated, while those determined in our experiments tend to fall short where they deviate from the calculated. The available data appear to agree with the predicted values as closely as the limitations of present accuracy in water determinations justify expecting.

In Fig. 2 the relative cell volume changes resulting from pH variations in oxygenated blood, as calculated by Equation 27, are shown for bloods of varying hemoglobin content. The percentage cell volume change caused by a given pH shift is greatest when the ratio cells:serum is least (hemoglobin lowest), because the concentration or dilution of serum which results from the water exchange, and tends to diminish the latter, is least when the relative amount of cells is smallest.

Illustration of the Effect of CO_2 Tension Changes on the Electrolyte and Water Distribution of Oxygenated Blood.

To illustrate the processes involved we may simplify conditions by ignoring minor factors; *viz.*, the slight amounts of diffusible anions other than Cl′ and HCO_3', the osmotic and base-binding powers of the serum proteins, and the osmotic effect of the hemoglobin. We shall assume the cells to contain only base, hemoglobin, Cl, and HCO_3, and the serum to be a simple solution of bicarbonate and chloride. Equation 10 under these conditions becomes simplified to

$$r = \frac{[H^+]_s}{[H^+]_c} = \frac{[Cl']_c}{[Cl']_s} = \frac{[HCO_3']_c}{[HCO_3']_s} = 1 - \frac{[BHb]_c}{2\left([BCl]_s + [BHCO_3]_s\right)}$$

We shall assume, first that the CO_2 tension is so low that $pH_c = 7.8$, then that it is raised so that pH falls to 6.6. According to Van Slyke, Hastings, Heidelberger, and Neill (1), the alkali bound by oxyhemoglobin is indicated by the equation $[BHb] = 2.65\ [Hb]\ (pH - 6.6)$. Assuming $[Hb]_c = 30$ mM., we therefore calculate at $pH_c = 7.8$ that $[BHb] = 95$, and at $pH_c = 6.6$ that $[BHb] = 0$.

In Fig. 3 we have indicated the concentrations of the positively and negatively charged ions in the cells and serum by the areas assigned to each ([Hb′] is indicated in terms of alkali equivalents bound). The concentrations of the osmotically active ions are indicated by clear areas, while that of the (relatively) osmotically

inactive [Hb′] is indicated by a shaded area. For simplicity it is assumed that the ionization of each electrolyte is complete. It is also assumed that at the beginning (Fig. 3 *A*) the water content of the blood is half in the cells, half in the serum.

The amounts of hemoglobin, base, chloride, and bicarbonate indicated are about those found in normal horse blood, except that the difference between $[B]_s$ and $[B]_c$ in Fig. 3 *A* is somewhat exaggerated as a result of ignoring the base bound by the serum proteins and the osmotic effect of the hemoglobin.

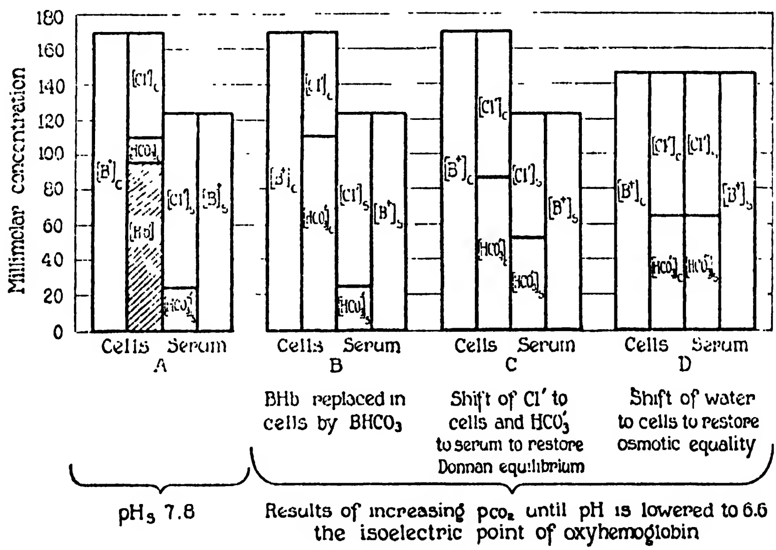

FIG. 3.

The conditions indicated in the four diagrams of Fig. 3 are the following:

A. The conditions represented conform to the three basic laws: (1) in both cells and serum the positive and negative ions balance; (2) the ratios $\frac{[Cl']_c}{[Cl']_s}$ and $\frac{[HCO_3']_c}{[HCO_3']_s}$ are equal, and conform to the simplified form of Equation 10 given above; and (3) the osmolar concentrations obtained by adding $[B^+] + [Cl'] + [HCO_3']$ are equal in serum and cells respectively.

B. Increase of CO_2 tension has lowered the pH_c to 6.6, the isoelectric point of oxyhemoglobin. The result is that all the base formerly bound by hemoglobin as BHb has shifted to $BHCO_3$, HCO_3' replacing Hb'. In Fig. 3 *B*, however, only the first of the three laws is conformed with. Positive and negative charges balance, but the greatly increased concentration of HCO_3' in the cells obviously makes $\frac{[HCO_3]_c}{[HCO_3]_s} > \frac{[Cl]_c}{[Cl]_s}$, contrary to Donnan's law. The HCO_3' increase in the cells also causes the osmolar concentration there to exceed that in the serum. The system is not in equilibrium.

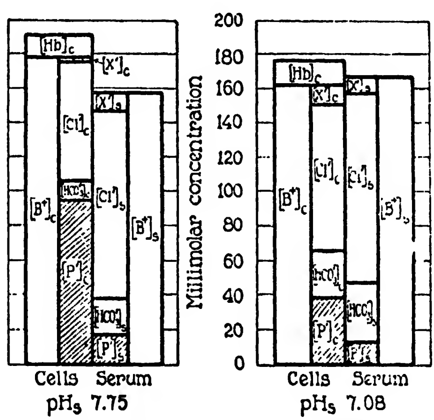

FIG. 4. Relationships observed in Experiment 3.

C. To restore electrolyte distribution to conformity with Donnan's law, Cl' has migrated from serum to cells, and HCO_3' in the reverse direction until again $\frac{[HCO_3]_c}{[HCO_3]_s} = \frac{[Cl]_c}{[Cl]_s}$.

D. To restore also osmotic equilibrium, water has migrated from serum to cells until the osmolar concentrations in both are equal. Impermeability of the cell membranes to cations prevents diffusion of BCl and $BHCO_3$ from cells to serum to assist in the restoration of osmolar equality. It must all be accomplished by water transfer. The system is now in equilibrium again.

The processes represented here, for the sake of analysis, as though occurring in successive steps must in reality occur simultaneously.

The somewhat more complex changes actually occurring in blood, where the alkali-binding power of the serum proteins and the osmotic pressure of the hemoglobin enter as appreciable, though minor factors, are indicated by Fig. 4, which represents data obtained in Experiment 3 on defibrinated blood. X′ is used to indicate the undetermined anions.

Calculation of the Electrolyte and Water Changes in Blood during the Respiratory Cycle.

In table III*a* we have calculated the changes that, according to our data, may be expected to accompany the CO_2 and O_2 changes of ordinary respiration. For a blood of (Hb) = 9.0 mM. per kg. the other blood constants $(B)_c$, $(B)_s$, $(P)_s$, G_b, and $(H_2O)_b$ are calculated from Equations 15, 16, 17, 29, and 30. The $(Cl)_b$ content is taken arbitrarily at 76, and the $(BHCO_3)_b$ in arterial blood is taken at 19.0. The combined oxygen (O_2) is assumed to be 8.8 mM. in arterial blood, and 6.0 in venous blood, and pH_s is assumed to be 7.43 in arterial blood, and 7.40 in venous blood. From these pH_s values the r and pH_c values are estimated from the curves of Fig. 1.

The $(BP)_c$ and $(BP)_s$ values are calculated from Equations 57 and 54 respectively (see experimental part of the paper). The increment in $(BHCO_3)$ when the arterial blood becomes venous is estimated equal to the decrease in $(BP)_c + (BP)_s$, the increase in bicarbonate being due to alkali taken from the other buffers to combine with H_2CO_3. (The diffusible buffers other than carbonates are neglected, as their effect is relatively slight.)

The distribution of water between serum and cells is calculated from Equations 23 and 24.

The distribution of Cl and HCO_3 is calculated as follows:

Letting A represent either Cl or HCO_3, we have from Equation 9 $\frac{[A]_c}{[A]_s} = r$. But $[A]_c = \frac{(A)_c}{(H_2O)_c}$ and $[A]_s = \frac{(A)_s}{(H_2O)_s}$. Hence $\frac{(A)_c}{(A)_s} = r\frac{(H_2O)_c}{(H_2O)_s}$. We represent the factor $r\frac{(H_2O)_c}{(H_2O)_s}$ by (r). Then, $(A)_c = (r)\ (A)_s$, $\frac{(A)_c}{(A)_c + (A)_s} = \frac{(r)}{1 + (r)}$, and $\frac{(A)_s}{(A)_c + (A)_s} = \frac{1}{1 + (r)}$.

TABLE III *a*.

Estimation of the Changes Occurring in the Normal Respiratory Cycle.

		Arterial.			Venous.			Changes.	
	Serum.	Cells.	Whole blood.	Serum.	Cells.	Whole blood.	Serum.	Cells.	Whole blood.
(Hb) *mM. per kg. blood*	0.0	9.0	9.0	0.0	9.0	9.0	0.0	0.0	0.0
$(P)_s$ *gm.* " " "	37.0	0.0	37.0	37.0	0.0	37.0	0.0	0.0	0.0
(B) *m.-Eq.* " " "	73.0	54.0	127.0	73.0	54.0	127.0	0.0	0.0	0.0
(H_2O) *kg.* " " "	0.464	0.316	0.780	0.460	0.320	0.780	−0.004	+0.004	0.0
(Cl) *m.-Eq.* " " "	51.2	24.8	76.0	50.0	26.0	76.0	−1.1	+1.1	0.0
(BP) " " " "	6.62	22.01	28.63	6.54	19.87	26.41	−0.08	−2.14	−2.22
$(BHCO_3)$ " " " "	12.79	6.21	19.00	13.96	7.26	21.22	+1.17	+1.05	+2.22
(H_2CO_3) " " " "	0.626	0.426	1.052	0.733	0.510	1.243	+0.11	+0.08	+0.19
Total CO_2 " " " "	13.42	6.63	20.05	14.69	7.77	22.46	+1.27	+1.14	+2.41
Combined O_2 *mM.* " " "	0.0	8.8	8.8	0.0	6.0	6.0	0.0	−2.8	−2.8
Dissolved O_2 " " " "			0.14			0.03			−0.1
Total O_2 " " " "			8.94			6.03			−2.9
p_{CO_2} *mm. Hg* " " "	41.6	41.6	41.6	49.2	49.2	49.2	+7.6	+7.6	+7.6
p_{O_2} " " " " "	100.0	100.0	100.0	38.0	38.0	38.0	−62.0	−62.0	−62.0
pH	7.43	7.283		7.40	7.272		−0.030	−0.011	
Volume *l. per l. blood*	0.525	0.475	1.000	0.521	0.479	1.000	−0.004	+0.004	0.0
r ratio			0.713			0.746			+0.033
(*r*) "			0.485			0.519			+0.034
Respiratory quotient $\frac{\Delta CO_2}{\Delta O_2}$									0.83

TABLE III *b.*

Changes from Arterial to Venous Blood.

	Total CO_2.	Cell volume.	Serum Cl.	Proportion of total CO_2 increase in venous cells.	Proportion of total CO_2 increase in venous serum.
	mM. per kg. blood	*per cent of blood vol.*	*mM. per l. serum*	*per cent*	*per cent*
Calculated from data of Table III *a* on horse blood..................	+2.4	+0.4	−1.2	49.3	50.7
Observed by Doisy and Beckmann (20) in dog blood..................	+1.9	+0.35	−1.38	52.4	47.6

From the values of (r), and of $(A)_c + (A)_s$, which is the total $(Cl)_b$ or $(HCO_3)_b$, we accordingly calculate $(A)_c$ and $(A)_s$.

Using the pK′ value 6.12 (see "Calculation" in experimental part), we calculate the $(H_2CO_3)_s$ and $(H_2CO_3)_c$ from the corresponding $(BHCO_3)$ and pH values by a rearrangement of Hasselbalch's (21) equation as

$$\log (H_2CO_3) = pK' - pH + \log (BHCO_3)$$

The CO_2 tension is calculated as

$$p_{CO_2} = \frac{(H_2CO_3)}{0.0324 \times (H_2O)}$$

since $[H_2CO_3] = \dfrac{(H_2CO_3)}{(H_2O)} = 0.0324\ p_{CO_2}$ (see "Calculations" in experimental part).

The oxygen tensions have been approximated from Barcroft's curves on human blood (37). Their degree of accuracy when applied to horse blood data is uncertain, but we have used them to indicate at least the magnitude of the changes.

In short the serum pH and the oxygen contents of the venous and arterial bloods have been arbitrarily chosen at about the observed normal values, and the other data have been calculated from them.

It will be noted that in Table III*a* the figures are given in terms of millimols or milli-equivalents of serum or cell constituents per kg. of whole blood, not per kg. or per liter of the serum or cells. The concentration values in the serum and cells in terms of solute: water may be obtained from the figures per kilo of blood by dividing the latter by the water content; *e.g.*, $\dfrac{\text{serum Cl}}{\text{serum } H_2O} = \dfrac{\text{serum Cl}}{\text{kg. blood}} \div \dfrac{\text{serum } H_2O}{\text{kg. blood}}$. Similarly $\dfrac{\text{serum Cl}}{\text{liter serum}} = \dfrac{\text{serum Cl}}{1.05 \text{ kg. blood}} \div \dfrac{\text{liter serum}}{\text{liter blood}}$.

In Table III*b* we have compared the average arterial-venous differences observed in dog blood by Doisy and Beckmann (20) with the changes calculated from Table III*a*. The observed figures are of the same order of magnitude as those calculated.

In Fig. 5 we have indicated the relationships on a D'Ocagne nomogram, of a type that was devised by L. J. Henderson (private communication about 2 years ago). A straight line, drawn across the chart, and cutting the lines representing oxygen and CO_2 tensions at any given points, cuts the lines representing $(BHCO_3)_b$, (HbO_2), pH_s, pH_c, etc., at points indicating the values these respective quantities have under the given p_{CO_2} and p_{O_2}. Such a line can be drawn because all the other variables in a given blood are dependent on these two. Over the range used, the chart is quite exact. The construction of such blood charts will be discussed in a later paper by Henderson.

Electrolyte Distribution between Blood Serum and Transudates as a Function of the Alkali Bound by the Proteins.

Loeb, Atchley, and Palmer (22) have performed experiments indicating that the membranes separating the blood serum from the fluids in the body cavities and intercellular spaces have the same permeabilities as collodion for the substances present. Under these conditions the Donnan distribution would require expression by an equation including Na and K among the diffusible ions, instead of excluding them, as does Equation 3. Expressing the distribution ratio of monovalent ions between serum and fluid as r_{sf}, the relationship would theoretically be

$$r_{sf} = \frac{[A']_s}{[A']_f} = \frac{[B^+]_f}{[B^+]_s} = \frac{[H^+]_f}{[H^+]_s} \tag{31}$$

when $\frac{[A']_s}{[A']_f}$ indicates the ratio of the osmotic activity of any monovalent anion, or sum of anions, in the serum to the osmotic activity of the same ion or ions in the fluid, while $\frac{[B^+]_f}{[B^+]_s}$ has a similar significance for the cations. If in place of $[B^+]_s$ and $[B^+]_f$ we substitute their values from Equations 1 and 2, we obtain

$$r_{sf} = \frac{[A']_f + [BP]_f}{[A']_s + [BP]_s} \tag{32}$$

If we substitute $\frac{A_s}{(r_{sf})}$ for A_f, and solve for r_{sf}, we obtain

$$(33)\qquad r_{sf} = \frac{[BP]_f + \sqrt{[BP]^2_f + 4\,[A]_s\,([A]_s + [BP]_s)}}{2\,([A]_s + [BP]_s)}$$

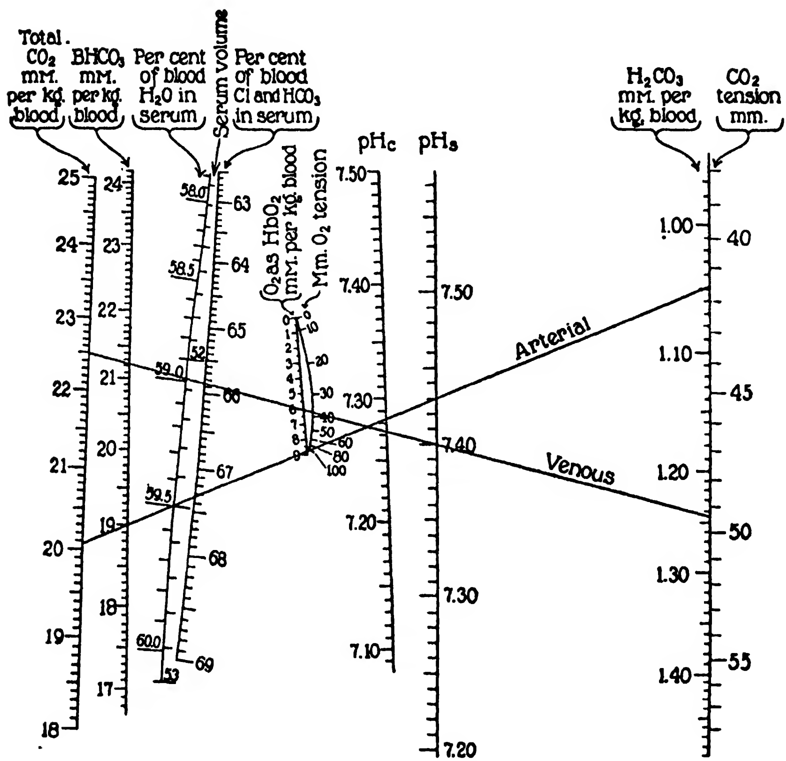

FIG. 5. D'Ocagne-Henderson nomogram showing calculated relationships for arterial and venous blood of average serum and cell composition observed in our experiments.

We have recalculated in Table IV Loeb, Atchley, and Palmer's data, transposing the concentrations from $\frac{\text{solute}}{\text{volume}}$ to $\frac{\text{solute}}{\text{water}}$ ratios by estimating the grams of water per liter to be 990 − 0.8 P, where P represents grams of protein per liter. (This may be taken as a fairly close approximation, unless abnormal amounts of fat or other

TABLE IV.

Observed Electrolyte Distribution between Blood Serum and Serum Cavity Fluids Compared with Distribution Ratio Calculated from Base Bound by Proteins. Calculated from the Data of Loeb, Atchley, and Palmer.

Subject	Cl	K	Co	D	P	McA	H
Extravascular fluid	Ascitic.	Ascitic.	Ascitic.	Chest.	Ascitic.	Ascitic.	Chest.
Serum protein, *gm./l.*	68	52	70	50	60	73	71
Fluid " "	9	8	9	6	33	45	56
Estimated serum H_2O, *kg./l.*	0.936	0.948	0.934	0.950	0.942	0.932	0.933
" fluid " "	0.982	0.984	0.983	0.985	0.964	0.954	0.945
$[P]_s$ *gm./kg. H_2O*	73	55	75	53	64	78	76
$[P]_f$ " "	9	8	9	6	34	47	59
$[BP]_s$ *m.-Eq./kg. H_2O*	12.9	9.7	13.3	9.4	11.3	13.9	13.5
$[BP]_f$ " "	1.5	1.4	1.5	1.0	6.0	9.3	10.0
$[Cl]_s$ " "	107.0	109.5	113.0	116.7	122.5	113.4	108.5
$[Cl]_f$ " "	110.2	110.8	115.7	121.5	125.6	118.0	110.5
Venous $[HCO_3]_s$ " "	29.4	25.1	28.3	28.4	17.9	30.5	32.0
Estimated arterial $[HCO_3]_s$ " "	27.4	23.1	26.3	26.4	15.9	28.5	30.0
$[HCO_3]_f$ " "	26.8	24.2	26.0	27.0	16.8	29.8	31.0
$[Na]_s$ " "	133.2	141.1	120.0	157.1	148.5	150.2	148.6
$[Na]_f$ " "	140.8	128.8	141.0	149.8	146.0	148.0	150.5
$[K]_s$ " "	4.5	5.0	2.9	3.7	5.0	5.0	4.7
$[K]_f$ " "	2.4	2.0	2.4	1.7	3.2	3.4	3.1
$[Cl]_s : [Cl]_f$ ratio	0.97	0.99	0.98	0.96	0.98	0.96	0.98
Venous $[HCO_3]_s : [HCO_3]_f$ "	1.10	1.04	1.08	1.05	1.07	1.02	1.03
Estimated arterial " "	1.02	0.95	1.01	0.98	0.95	0.96	0.97
$[Na]_f : [Na]_s$ "	1.06	0.91	1.17	0.95	0.98	0.99	1.01
$[K]_f : [K]_s$ "	0.53	0.40	0.83	0.46	0.64	0.68	0.66
Calculated r_{sf}	0.95	0.97	0.96	0.96	0.98	0.98	0.90

solids are present.) The $[BP]_s$ and $[BP]_f$ values are calculated on the assumption that the proteins of human serum at pH 7.4 bind the same amount of alkali per gram as the proteins of horse serum (the slight difference in pH between plasma and fluid may be neglected). At this pH the formula (Equation 54) $[BP] = 0.068\ [P]\ (pH - 4.80)$ becomes $[BP] = 0.177\ [P]$. The arterial HCO_3 values are estimated by subtracting 2 mM. per liter from the values found in the venous serum.

The estimated $[Cl]_s:[Cl]_f$ ratios found coincide with the calculated r_{sf} values nearly within the limit of experimental error. The $[HCO_3]_s:[HCO_3]_f$ ratios are all higher than the calculated r_{sf} when the venous values for $[HCO_3]_s$ are used; but the estimated arterial values for $[HCO_3]_s$ yield $[HCO_3]_s:[HCO_3]_f$ ratios which agree with the calculated r_{sf} as closely as could be expected, when the possible magnitude of the error involved in assuming a constant difference between arterial and venous CO_2 is considered.

The $[Na]_f:[Na]_s$ ratios agree, in six out of seven cases, with the calculated r_{sf} values within the rather wide limit of error assigned by the authors to the Na determination. The $[K]_f:[K]_s$ ratios are altogether lower than the calculated r_{sf}, and are very irregular. The source of the deviation and irregularity of the K ratios is at present uncertain. Considering the minute amounts of K present, it appears possible that the irregularities may lie in the micro method used for the determination.

The irregularity of the potassium ratios, and the necessity for using estimated water and arterial HCO_3 values, make it impossible to consider the presence of a Donnan equilibrium between blood serum and edema fluid as quantitatively demonstrated with satisfactory accuracy. It appears probable, nevertheless, that the degree of agreement found between the calculated r_{sf} values and the ratios for Cl, HCO_3 (arterial), and Na is more than fortuitous; that it affords support for Loeb, Atchley, and Palmer's conclusion that "the relationships between serum and edema fluid result from a simple membrane equilibrium, influenced in part by the proteins present."

If the membranes separating blood serum from other extracellular fluids are permeable to all electrolytes present in amounts of quantitative importance except protein, it follows from the Donnan theory

that the serum, containing more protein than the other fluids, must when at equilibrium with them show a positive osmotic pressure. While the basic equations of the form of Equations 1 and 2, and of Equation 3 modified to include Na, hold for such a system, Equation 4 and its derivatives expressing osmolar equality do not, so long as the serum volume is limited. The preponderance of the osmolar concentration even of the diffusible ions, on the side containing non-diffusing ions, when the latter are entirely on one side of the membrane and infinite volume change is excluded, has been theoretically shown by Procter and Wilson (15).

If the non-diffusible electrolyte (protein) also has a measurable attraction for water, the osmotic preponderance on its side of the membrane is still further increased. If serum and a transudate relatively poor in protein are separated by membranes permeable to all the non-protein ions present in quantitatively important concentrations, *viz.* Na^+, Cl', and HCO_3', but impermeable to the protein, we may therefore expect the serum to exhibit a higher osmotic pressure than the edema fluid. With the osmotic pressure tending to draw water into the serum, it appears that forces other than that of osmotic pressure are involved in the passage of fluid in the direction from the blood to the serous cavities and intercellular spaces.

Effect of the Membrane on the Determination of the Blood pH by the Dialysis Method.

Levy, Rowntree, and Marriott (23) have devised a method for blood pH determination in which the blood is dialyzed against 0.8 per cent NaCl solution, and the pH of the dialysate is determined colorimetrically. This method has later been revised by Dale and Evans (24), who added precautions to prevent loss of CO_2. It is evident that, because protein is present only on one side of the membrane, the Donnan effect must introduce some error into such a method. On the basis of our data defining the amount of base bound by the serum proteins at physiological pH ranges, it appears possible to estimate the approximate magnitude of this error.

In the short time in which measurable change of pH in the dialysis fluid ceases (5 minutes) the volume change may be neglected, and we

may consider the system the same as that discussed in the preceding section, *viz.* one in which the volumes are fixed, the osmotic pressures are not necessarily equal, and the solutes, except protein, are diffusible through the membrane. Equation 33 then applies but is simplified because $[BP]_f$ is zero, since there is no protein in the fluid outside the membrane. In place of Equation 33 we therefore have, when equilibrium among the electrolytes is reached:

$$(34) \qquad r_{s,f} = \frac{[H^+]_f}{[H^+]_s} = \sqrt{\frac{[A]_s}{[A]_s + [BP]_s}}$$

whence

$$(35) \qquad pH_f - pH_s = \frac{1}{2} \log \frac{[A]_s + [BP]_s}{[A]_s}$$

If we take $A_s = 150$ mM., $[BP]_s = 14$, which are fairly close to the usual values, we obtain

$$pH_f - pH_s = \frac{1}{2} \log \frac{164}{150} = 0.02$$

It appears, therefore, that when serum (or blood) is dialyzed against a solution of approximately the same salt concentration, we may expect a pH in the solution about 0.02 greater than in the serum.

Relationship between Hasselbalch's Constant for Whole Blood and the K'_s Value for Serum.

As Warburg has pointed out, since blood is a heterogeneous system, the cell and serum pH and $BHCO_3$ values differ, the constant K'_B of the equation

$$(36) \qquad K'_B = [H^+]_s \frac{[BHCO_3]_b}{[H_2CO_3]_b}$$

used by many authors since Hasselbalch (20) in applying Henderson's equation to whole blood, is really an approximate constant, which contains within itself corrections, hitherto empirical, for the differences that exist between cells and serum. (Hasselbalch used the symbol K_1. We have used K'_B in order to distinguish it from K'_s.) The relationship of K'_B to the constant K'_s is shown as follows:

$$(37) \qquad K'_s = [H^+]_s \frac{[BHCO_3]_s}{[H_2CO_3]_s}$$

Dividing Equation 36 by Equation 37, we obtain

$$\text{(38)} \qquad \frac{K'_B}{K'_s} = \frac{[BHCO_3]_b}{[BHCO_3]_s} \times \frac{[H_2CO_3]_s}{[H_2CO_3]_b}$$

If concentrations are expressed in the terms of solute : water ratios used in this paper $[H_2CO_3]_s$ and $[H_2CO_3]_b$ are equal (see discussion of $[H_2CO_3]$ calculations on pages 415 to 417). Under these conditions $\frac{[H_2CO_3]_s}{[H_2CO_3]_b} = 1$, and Equation 38 simplifies to

$$\text{(39)} \qquad \frac{K'_B}{K'_s} = \frac{[BHCO_3]_b}{[BHCO_3]_s}$$

Equation 38 may be used for calculating $\frac{K'_B}{K'_s}$ from the CO_2 contents of whole blood and true serum, whatever unit of concentration is employed, and Equation 39 may be used with the solute : water ratio adopted as the concentration unit in this paper.

The relationship of the $\frac{K'_B}{K'_s}$ ratio to the electrolyte and water distribution in the blood is shown as follows:

Since the total bicarbonate in 1,000 gm. of blood is equal to the sum of that in the serum plus that in the cells, we have

$$\text{(40)} \qquad [BHCO_3]_b \times (H_2O)_b = [BHCO_3]_s \times (H_2O)_s + [BHCO_3]_c \times (H_2O)_c$$

whence

$$\text{(41)} \qquad [BHCO_3]_b = [BHCO_3]_s \frac{(H_2O)_s}{(H_2O)_b} + [BHCO_3]_c \frac{(H_2O)_c}{(H_2O)_b}$$

but $[BHCO_3]_c = r\,[BHCO_3]_s$ (see Equation 3).

Substituting in Equation 41 the above value for $[BHCO_3]_c$, we obtain

$$\text{(42)} \qquad [BHCO_3]_b = [BHCO_3]_s \frac{(H_2O)_s + r\,(H_2O)_c}{(H_2O)_b}$$

Substituting this value for $[BHCO_3]_b$ in Equation 39, we obtain

$$\text{(43)} \qquad \frac{K'_B}{K'_s} = \frac{(H_2O)_s + r\,(H_2O)_c}{(H_2O)_b}$$

In logarithmic terms, using $pK'_B = -\log K'_B$, $pK'_s = -\log K'_s$, Equation 43 becomes

$$pK'_B - pK'_s = \log \frac{(H_2O)_b}{(H_2O)_s + r\,(H_2O)_c} \qquad (44)$$

The values for $(H_2O)_s$ and $(H_2O)_c$ are obtainable from Equations 23 and 24, and the values of r from Equations 10 and 12.

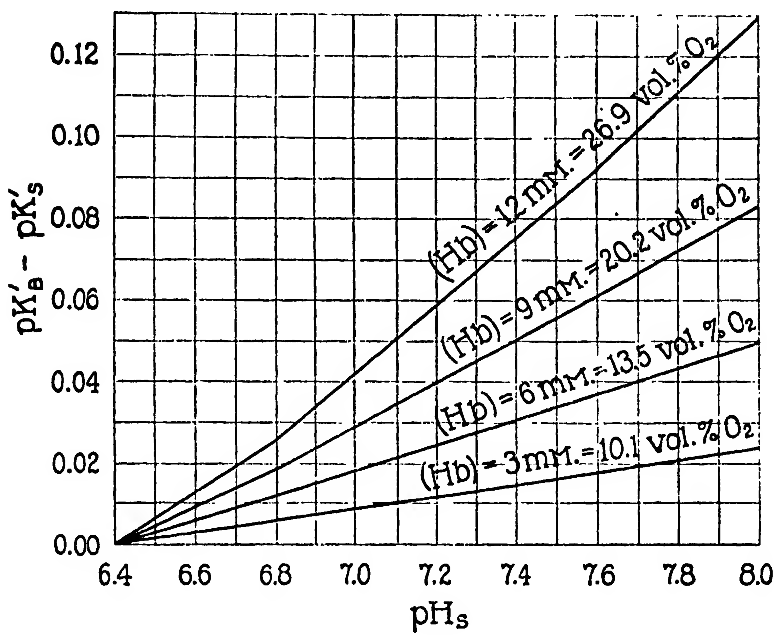

FIG. 6*a*.

The values of $pK'_B - pK'_s$ calculated by Equation 44 at varying pH_s and for bloods of varying hemoglobin content are given in Fig. 6*a*.

In Fig. 6*b* Dr. Hastings has prepared a D'Ocagne line chart which facilitates interpolations of the Hb, pH_s, and $pK'_B - pK'_s$ relationships. The chart was constructed by laying off the (Hb) and $pK'_B - pK'_s$ values logarithmically on their respective lines, and locating each pH_s point at the intersection of the lines, connecting the 3, 6, 9, and 12 (Hb) points with the $pK'_B - pK'_s$ points calculated from

the given pH_s and the respective (Hb) values. Over the Hb range 3 to 9 mM., the chart indicates values for $pK'_B - pK'_s$ differing from those calculated from Equation 44 by only 0.001. A straight line drawn across the chart indicates the $pK'_B - pK'_s$ value corresponding to the Hb and pH_s points intersected.

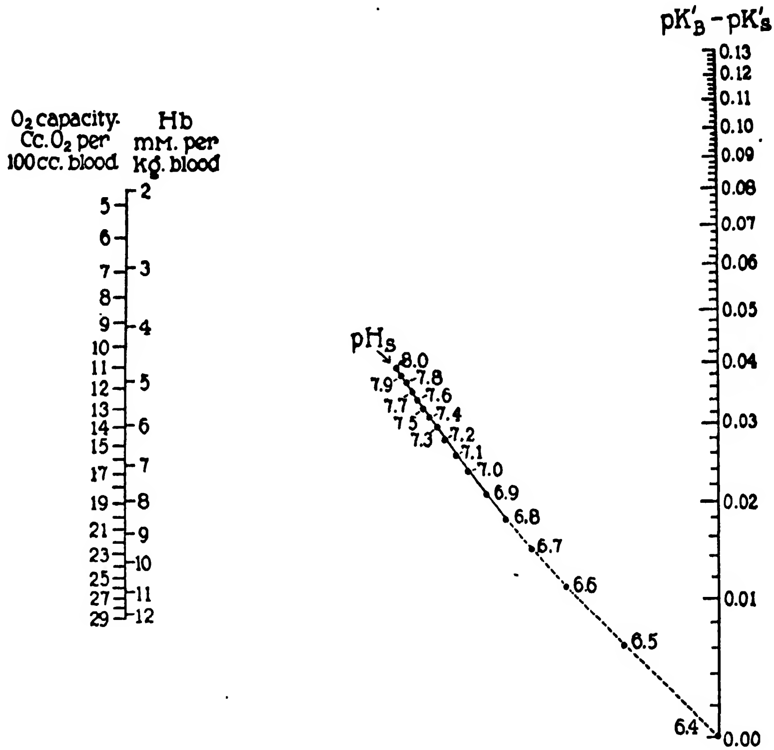

FIG. 6*b*.

The curves are of the same form as those obtained in a different manner by Warburg.[6] The values for $pK'_B - pK'_s$ indicated by our curves are 0.01 to 0.02 higher than Warburg's at ordinary physiological pH and (Hb) ranges.

Peters, Bulger, and Eisenman (25), in a paper appearing since our work was completed, have determined the value of $pK'_B - pK'_s$

[6] See Warburg (10), p. 220.

(for which they use the symbol Δ pK_1) in the most direct way from determinations of the CO_2 contents of whole blood and plasma in a large series of human bloods, saturated at known CO_2 tensions, their calculation being made practically as by our Equation 38. Their Δ pK_1 values, obtained within the range of their experiments (indicated by the solid lines of their Chart 4), when transposed from cell volume to (Hb) terms, deviate from our curves by 0.000 to 0.009. Considering the facts that their data represent the average from many human bloods, while ours are from the blood of one horse, and also that the difference between Bohr's solubility coefficients for CO_2 used by them and the coefficients used by us appreciably affect the Δ pK_1 values, the agreement is close.

Calculation of the CO_2 Absorption Curve and Buffer Value of Blood.

Since, in a given blood submitted to varying CO_2 tensions, the changes in bicarbonate are at the expense of alkali taken from or given to the other buffers (the proteins), we may represent the total blood bicarbonate $(BHCO_3)_b$ as

$$(45) \qquad (BHCO_3)_b = (B)_{P+HCO_3} - (BP)_b$$

where $(B)_{P+HCO_3}$ is a constant representing the base bound by proteins and bicarbonate together. (In normal horse blood, approximately $(B)_{P+HCO_3} = (B)_b - 1.1\ (Cl)_b$.) Since $(BP)_b = (BP)_c + (BP)_s$ we may write Equation 45 as

$$(46) \qquad (BHCO_3)_b = (B)_{P+HCO_3} - \left\{(BP)_c + (BP)_s\right\}$$

By substituting from Equations 54 and 55 the approximate values for $(BP)_s$ and $(BP)_c$ in oxygenated blood, we obtain $(BHCO_3)_b$ in terms of pH and protein content

$$(47) \qquad (BHCO_3)_b = (B)_{P+HCO_3} - \left\{3.6\ (Hb)\ (pH_c - 6.6) + 0.068\ (P)_s\ (pH_s - 4.80)\right\}$$

The pH values may all be reduced to pH_s by substituting pH_s + $\log r$ for pH_c (see Equation 18). We thus obtain

$$(48) \qquad (BHCO_3)_b = (B)_{P+HCO_3} - \left\{3.6\ (Hb)\ (pH_s + \log r - 6.6) + 0.068\ (P)_s\ (pH_s - 4.80)\right\}$$

Between pH 7.0 and 7.8 we may approximate the value of log r (see Fig. 1) with an error not greater than 0.01 by the linear formula,

$$(49) \qquad - \log r = 0.21 \text{ pH}_s - 1.41$$

Substituting this value for log r in Equation 48, we obtain

$$(50) \qquad (BHCO_3)_b = (B)_{P + HCO_3} - \{2.84 \text{ (Hb) } (\text{pH}_s - 6.6) + 0.068 \text{ (P)}_s (\text{pH}_s - 4.80)\}$$

Equation 50 represents the CO_2 absorption curve of oxygenated blood with $(BHCO_3)_b$ and pH_s as ordinates.

(The same method of calculation may be used for reduced blood, 3.35 (Hb) being substituted for 3.6 (Hb), and $- \log r = 0.18 \text{ pH}_s - 1.15$ for the above $- \log r$ value. See Fig. 1 and Equation 55.)

By differentiating Equation 50 with respect to pH_s we obtain the $\frac{d(BHCO_3)_b}{d\text{pH}_s}$ value, which is the negative of the buffer values other than that of bicarbonate.

$$(51) \qquad - \frac{d(BHCO_3)_b}{d\text{pH}_s} = 2.84 \text{ (Hb)} + 0.068 \text{ (P)}_s$$

That Equation 51 is approximately accurate for normal horse blood is indicated by comparison with the buffer values obtained on whole blood in the fourth paper (2) of this series and summarized in Table VII of that paper. Estimating the $(P)_s$ values for the bloods from the hemoglobin contents by Equation 17 of the present paper, and calculating the buffer values by Equation 51, we obtain the results below.

Blood No. (Table VII Van Slyke, Hastings, and Neill (2))	$-\frac{d(BHCO_3)_b}{d\text{pH}_s}$ Observed.	Calculated by Equation 51.
1	23.72	23.9
2	20.64	22.6
4	24.18	23.5
5	22.23	23.0
6	24.16	24.9

If the entire blood buffer system (as in laked blood) were at pH_s, we should have the $(BHCO_3)_b$ represented by Equation 48 with log r deleted from it, and differentiation would give

$$(52) \qquad -\frac{d(BHCO_3)_b}{dpH_s} = 3.6\ (Hb) + 0.068\ (P).$$

The difference between Equations 51 and 52, *viz.* 0.76 (Hb), indicates the difference between the $\frac{d(BHCO_3)_b}{dpH_s}$ value estimated on the assumption that all the blood buffers are at pH_s and the value estimated on the assumption that the cell buffers are at $pH_c = pH_s + \log r$, or the approximate *increase in buffer value caused by laking blood.* (The value 0.76 (Hb) is only an approximation, because of the simplifying assumptions of linear functions that have been made above in deriving it.)

In the fourth paper of this series (2) the statement has been made that "The estimation from buffer constants obtained on hemoglobin solutions, of the buffer effect of hemoglobin in the blood involves the assumption that the confinement of hemoglobin within the red cells does not alter its buffer effect on the system. . . . . The calculation of the relative part played by hemoglobin in the buffer effect of blood . . . . is therefore presented with the reservation that slight corrections may be required because of the difference in pH between plasma and cell contents." It is obvious from the difference between Equations 51 and 52 above that confinement within the cells does alter the effect of the cell buffers on the system, and that the corrections anticipated as a possibility are of considerable significance. The point will be discussed in another paper with experimental data.

Equations 48, 50, and 51 as approximations indicate a straight line $(BHCO_3)_b$, pH_s curve for blood, and an approximately linear curve is observed in blood, as shown by the results of Warburg (10) and Van Slyke, Hastings, and Neill (2). However, the $(BP)_c$ value deviates slightly from the linear pH_c function assumed in Equation 52 (see Fig. 10), and the r value deviates considerably from a linear pH_s function (see Fig. 1). Both deviations tend to make the $(BHCO_3)_b$, pH_s curve assume towards the pH_s axis a convexity that increases with increasing hemoglobin content.

In Fig. 7 are shown the $(BHCO_3)_b$, pH_s curves for blood with (Hb) = 3, 6, 9, and 12 mM., as calculated by Equations 45 and 46. The values of $(BP)_c$ are estimated as follows: pH_c is obtained by subtracting $- \log r$ from pH_s, the $- \log r$ value being obtained from the curve of Fig. 1. With this pH_c value, the $(BP)_c$ value per millimol of hemoglobin is obtained from the curve of Fig. 10. This value multiplied by (Hb) gives $(BP)_c$ for the blood in question. $(BP)_s$ is calculated by Equation 54, *viz.* $(BP)_s = 0.068\ (P)_s\ (pH_s - 4.80)$,

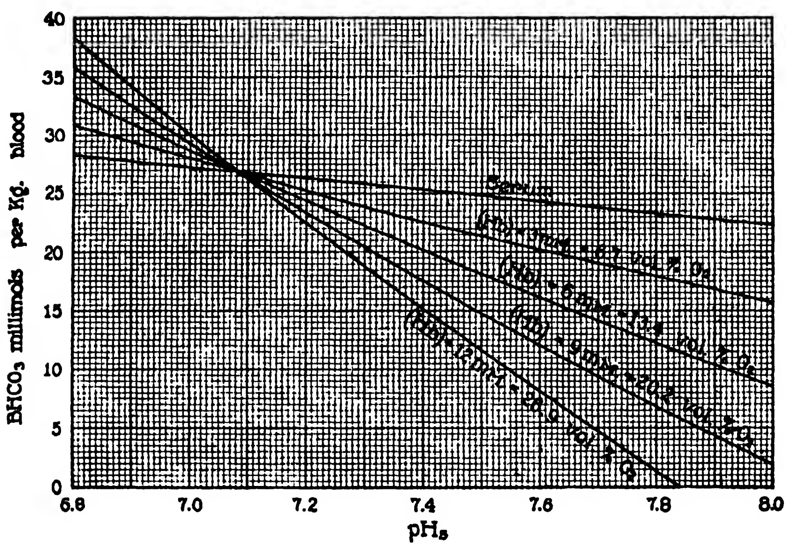

FIG. 7. Carbon dioxide absorption curves calculated by Equations 50 and 53 for oxygenated horse blood of average serum and cell composition observed in our experiments.

$(P)_s$ being estimated from Equation 17. The constant $(B)_{P+HCO_3}$ we have estimated from our data in Tables VIII to XI by the method employed in deriving Equations 15 and 16, which yields the equation

$$(B)_{P+HCO_3} = 38 + 0.8\ (Hb) \tag{53}$$

Like the other numerical constants derived in this manner, these values of $(B)_{P+HCO_3}$ represent only the blood of the individual Mongolian pony which donated the material for these experiments. The figures are used to indicate merely the relative magnitude of the

values concerned. Other normal bloods even of the same species, may differ considerably. The horse used by Van Slyke, Hastings, and Neill (2), for example, had blood with a $(P)_{P+HCO_3}$ value about 5 milli-equivalents lower than that of our animal. The levels of observed curves in samples of normal horse blood may therefore vary considerably, and the slopes somewhat, from those of the curves of Fig. 7, which are given only as illustrations of the forms that may be expected in bloods of varying hemoglobin content.

EXPERIMENTAL.

The Alkali-Binding and Buffer Values of the Proteins of Horse Serum.

Solutions of serum proteins were prepared by dialysis of serum. Both the serum and the water against which it was dialyzed were saturated with CO_2 in order to remove the alkali from combination with the proteins, a device originated by Adolph and Ferry (26). As low a pH as can be obtained with CO_2 is especially necessary to remove the alkali from combination with the serum proteins because their isoelectric pH points are so low, about 4.7 for the albumin and 5.4 for the globulin according to Michaelis (13). The dialysis was performed in closed collodion sacs as suggested by Adair, Barcroft, and Bock (27), and was continued until the conductivity had been lowered to such a degree as to indicate an electrolyte concentration of less than 0.001 N. During the dialysis a considerable part of the globulins precipitated out. They were suspended in the solution, and the suspension was transferred to 100 cc. flasks in amounts slightly less than enough to fill the flasks. 3 cc. of standard 0.5 M Na_2CO_3 and 600 mg. of NaCl were added, and a perfectly clear solution was obtained. The latter was made up to 100 cc. volume and used for determinations of the carbon dioxide-binding power at various CO_2 tensions according to the technique utilized by Van Slyke, Hastings, Heidelberger, and Neill (1).

In one detail of convenience our technique differed from that utilized by them. Instead of using the gas burettes indicated in Fig. 3 of their paper, the inlet cocks were connected directly with the air and a Kipp CO_2 generator, and a manometer was attached to

the system. After the tonometer had been evacuated to 700 mm., CO_2 was let in until the manometer registered the desired pressure change. The desired initial CO_2 tension could thus be measured directly with a sufficient degree of approximation for the present experiments.

Calculations.—The H_2CO_3 values were calculated, for reasons discussed under "Methods of calculation" on p. 415, on the assumption that with varying protein content the CO_2 solubility is proportional to the water content of the solution. Since the serum solutions were made up with approximately the same salt concentration as the original serum, the same solubility factor is used. Hence

$$\text{mM. } H_2CO_3 \text{ per liter solution} = 0.0324 \times kg.\ H_2O \text{ per liter solution} \times p_{CO_2}$$

The pH values are calculated according to Hasselbalch's equation $pH = pK' + \log \frac{BHCO_3}{H_2CO_3}$. The value used for pK′ is 6.12. The reasons for the choice of this value, on the basis of the experimental data of Cullen (28), are given on p. 417.

Two preparations of serum proteins were used, with the results given in Tables V and VI, and in Fig. 8. From the mean of the two linear curves obtained the following formula is derived.

$$\text{(54)} \qquad \text{mM. } [BP]_s = 0.068\ [P]_s\ (pH_s - 4.80)$$

where mM. $[BP]_s$ represents milligram equivalents of alkali combined with protein, $[P]_s$ the grams of protein present.

The Alkali-Binding and Buffer Values of the Non-Diffusible Constituents of the Blood Cells.

The same technique employed with serum was utilized with washed cells. An unexpected difficulty was encountered, however. It was found that the cell solution, even after a fortnight's dialysis, consumes its own oxygen and produces similar amounts of CO_2 at such a rate as to make accurate results impossible. Within 2 hours after the cell solutions had been saturated with air plus CO_2 practically all of their oxygen had disappeared, the solutions had become black, and an equivalent increase in CO_2 had occurred. Error from pro-

TABLE V.

Alkali-Binding Power of Dialyzed Horse Serum.

N content of solution	8.05 gm. per l.
Protein content = N × 6.25	50.25 " " "
Water content	0.950 kg. per l.
Factor for calculating mM. H_2CO_3 per l. from p_{CO_2}	0.0308
Conductivity at 25°	5.8 × 10^{-5} mhos.
Na added as Na_2CO_3	30.53 m.-Eq. per l.
" " " NaCl	100.0 " " "

Saturation Data.

No.	CO_2 tension.	Total CO_2.	H_2CO_3	$BHCO_3$	BP	$\frac{BP}{P}$	pH
	mm.Hg.	*mM. per l.*	*mM. per l.*	*mM. per l.*	*m.-Eq. per l.*	*m.-Eq. per gm. protein*	
1	16.0	21.15	0.493	20.66	9.87	0.1964	7.743
2	39.0	23.11	1.201	21.91	8.62	0.1715	7.380
3	70.2	24.80	2.163	22.63	7.90	0.1573	7.140
4	83.8	25.50	2.581	22.92	7.61	0.1515	7.068

TABLE VI.

Alkali-Binding Power of Dialyzed Horse Serum.

N content of solution	7.55 gm. per l.
Protein content = N × 6.25	47.2 " " "
Water content	0.952 kg. per l.
Factor for calculating mM. H_2CO_3 per l. from p_{CO_2}	0.0309
Conductivity at 25°	5.8 × 10^{-5} mhos.
Na added as Na_2CO_3	30.60 m.-Eq. per l.
" " " NaCl	100.0 " " "

Saturation Data.

No.	CO_2 tension.	Total CO_2.	H_2CO_3	$BHCO_3$	BP	$\frac{BP}{P}$	pH
	mm.Hg.	*mM. per l.*	*mM. per l.*	*mM. per l.*	*m.-Eq. per l.*	*m.-Eq. per gm. protein*	
1	16.9	21.60	0.522	21.08	9.52	0.2018	7.726
2	31.7	23.03	0.980	22.05	8.55	0.1812	7.472
3	59.7	24.53	1.845	22.68	7.92	0.1678	7.209
4	67.8	25.00	2.097	22.90	7.70	0.1632	7.158

duction of CO_2 was then obviated by starting the CO_2 determination within 4 minutes after saturation was completed, and making a correction for the slight amount of CO_2 formed in that time. However, the proteins themselves appear to be affected by oxidation during the saturation, and perhaps during the dialysis, and to lose some of their alkali-binding power. Some loss of oxygen-binding

TABLE VII.

Base Bound by Dialyzed Blood Cells.

Total hemoglobin content (Stadie colorimetric)	5.53 mM. per l.
Oxygen capacity	5.31 " " "
$\frac{\text{Oxygen capacity}}{\text{Total hemoglobin}}$	0.960
H_2O	0.9085 kilos per l.
Factor for calculating H_2CO_3 from p_{CO_2}	0.0295
Conductivity	2.3×10^{-4} mhos.
KOH added	30.00 mM. per l.
KCl "	100.0 " " "

Saturation Data.

No.	pCO_2	Total CO_2.	Total O_2.	H_2CO_3	$BHCO_3$	BP	pH	$\frac{dB}{dHbO_2}$	BP estimated for completely reduced solution.		BP estimated for oxygenated solution.
	mm. Hg	*mM. per l.*	*mM. per l.*	*mM. per l.*	*mM. per l.*	*m.-Eq. per l.*			*m.-Eq. per l.*	*Eq. per mol Hb*	*Eq. per mol Hb*
1	192.6	34.22	0.04	5.68	28.54	1.46	6.820	0.47	1.44	0.260	0.73
2	148.5	30.95	0.13	4.38	26.56	3.44	6.902	0.52	3.38	0.612	1.13
3	106.5	28.03	0.12	3.14	24.89	5.11	7.016	0.57	5.04	0.912	1.48
4	78.6	25.28	0.13	2.32	22.96	7.04	7.115	0.61	6.96	1.258	1.87
5	56.8	22.76	0.03	1.68	21.08	8.92	7.218	0.63	8.90	1.608	2.24
6	41.8	20.52	0.07	1.23	19.29	10.71	7.315	0.66	10.65	1.926	2.59
7	29.1	18.38	0.09	0.859	17.52	12.48	7.429	0.67	12.42	2.245	2.92
8	20.5	16.04	0.13	0.605	15.43	14.57	7.526	0.70	14.48	2.620	3.32

power (methemoglobin formation?) also occurs during dialysis. We consequently performed both dialysis and CO_2 capacity determinations on reduced solutions, and made the dialysis as brief as possible. The cell solution was saturated with pure CO_2 and dialyzed in narrow collodion tubes at 20°C. for 2 days against a great volume (100 liters) of distilled water, which was agitated by a stirrer. The water

was changed every 12 hours. We attempted in this manner to attain the necessary purification with as short a dialysis as possible.

In the 2 day period we did not succeed in lowering the conductivity so far as in the longer dialyses used with the serum proteins. A choice had to be made, however, of a dialysis period such that the error due to alteration of hemoglobin should not be greater than that due to retained alkali.

The saturators were filled with mixtures of hydrogen and CO_2 instead of air and CO_2. In order to make reductions as complete as possible the solution (10 cc.) was introduced into the saturator, and the latter

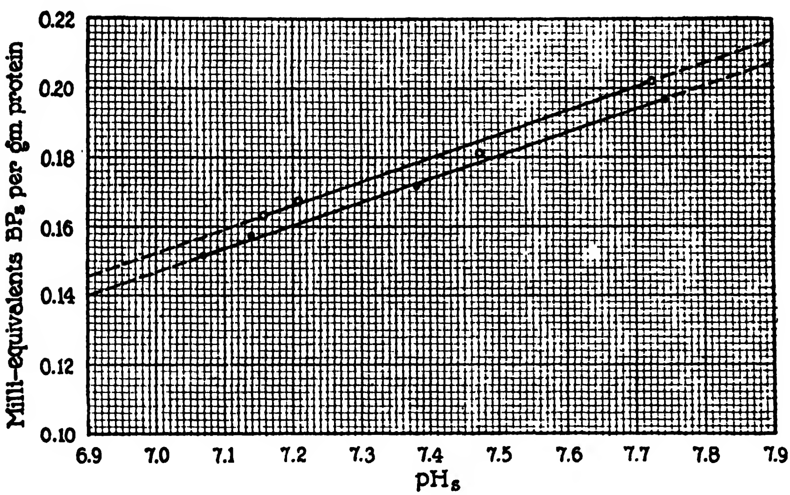

FIG. 8. Equivalents of base bound per gram of serum protein.

was evacuated, filled with hydrogen, and evacuated again. It was then rotated, so that the blood was distributed about the walls and the oxygen could be removed. The saturator was refilled with hydrogen and evacuated with a water pump three successive times to reduce the oxygen content to as nearly zero as possible, and was finally filled with the desired mixture of CO_2 and H_2. The hydrogen was supplied by a Kipp generator, and was passed through pyrogallol solution and water. The deoxygenation of the solutions was almost complete. The slight amounts of oxygen left were determined and are indicated in Table VII.

Solutions obtained in this manner did not form detectible amounts of CO_2 even in 2 hours standing after they had been saturated. Oxidation effects on the proteins, or other non-diffusible, alkali-binding substances in the cell solution, appear also to have been minimized.

During the limited period in which our experiments had to be completed at Peking we succeeded in ascertaining the precautions outlined for determining the base-binding power of dialyzed cell constituents, but time then remained only for two preliminary experiments on limited volumes of solutions, one of the latter being unusually dilute. In order to avoid delay in obtaining the necessary confirmation of the results, Hastings and Harington have come to

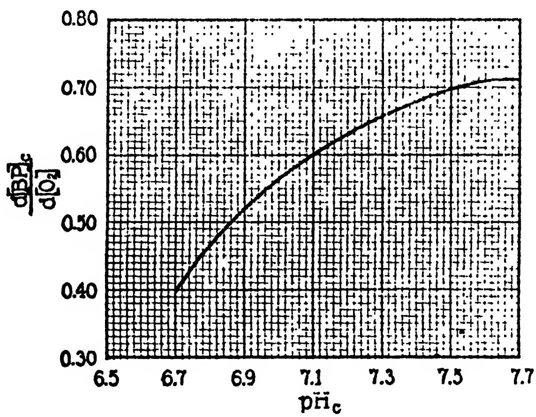

FIG. 9.

our assistance by preparing in the mean time cell solutions by the same technique at the Hospital of The Rockefeller Institute and determining the base-binding power. The results obtained by Hastings and Harington are given in Table VII and in Fig. 10.

Calculations.—The H_2CO_3 and pH values are calculated as described in connection with the experiments with the serum protein solutions. The figures for completely reduced cell contents are calculated as follows from those determined on the not quite entirely reduced solutions. On the basis of experiments by Hastings, Van Slyke, Neill, Heidelberger, and Harington[7] we have plotted in Fig. 9

[7] Hastings, A. B., Van Slyke, D. D., Neill, J. M., Heidelberger, M., and Harington, C. R., unpublished data.

the values of $\frac{d[BP]_c}{d[HbO_2]}$, the ratio indicating the increase in equivalents of alkali bound by the proteins per molecule of oxygen added to reduced hemoglobin. For each solution analyzed the decrease in base bound by the protein that would result from complete reduction of the hemoglobin is calculated as $[HbO_2] \times \frac{d\,[BP]_c}{d\,[HbO_2]}$. In these solutions the entire $[O_2]$ is calculated as $[HbO_2]$, since the amount of oxygen physically dissolved at the low oxygen tensions used was negligible. The portion of hemoglobin that had lost its oxygen-binding power during dialysis is assumed in this calculation to have the base-binding power of reduced hemoglobin, in accordance with the results of the above authors.

From the $[BP]_c$ figure for the reduced solution, that for the completely oxygenated solution is estimated by adding, to the $[BP]_c$ value per millimol of hemoglobin found for the completely reduced solution, $1 \times \frac{d\,[BP]_c}{d\,[HbO_2]}$, since in this case $\Delta\,[HbO_2]$ is 1, 1 molecule of oxygen being added to the reduced hemoglobin.

As a further check on our $[BP]_c$ curve, we have, in the three of the four experiments with defibrinated blood, described below, in which complete analyses were performed, estimated the $[BP]_c$ values as follows: We may write

$$[BP]_c = [B]_c - ([BCl]_c + [BHCO_3]_c + [BX]_c)$$

where $[BX]_c$ indicates the concentration of alkali bound to diffusible anions other than Cl and HCO_3. $[BX]_c$ is small, only about 0.1 as great as [BCl], and we have estimated it by assuming that $\frac{[BX]_c}{[BX]_s} = \frac{[BHCO_3]_c}{[BHCO_3]_s}$; hence $[BX]_c = [BX]_s \frac{[BHCO_3]_c}{[BHCO_3]_s}$. $[BX]_s$ was calculated as $[BX]_s = [B]_s - ([BCl]_s + [BHCO_3]_s + [BP]_s)$. Such calculations involve the summation of a number of analytical errors, and cannot be expected to be very exact. Nevertheless, the results calculated as described from analyses of defibrinated blood and shown on Fig. 10 by hollow circles, solid circles, and hollow squares, follow the curve

obtained from dialyzed cells plus $KHCO_3$ + KCl so closely that the evidence seems good that the curve represents fairly closely the amounts of alkali bound by the cell proteins in the blood.

The data from the experiment with dialyzed cells are given in Table VII, and, together with the data from the experiments with whole blood, in Fig. 10.

It will be seen that the oxygenated curve is slightly concave towards the pH axis, but it so nearly approximates a straight line that within the range covered there is little error in expressing it as such. Expressed by an approximate linear equation, the relationships may be indicated, *within the pH range of our data*, as follows:

For oxygenated blood,

$$[BP]_c = 3.6\,[Hb]_c\,(pH_c - 6.6) \tag{55}$$

For reduced blood, representing reduced hemoglobin also as Hb,

$$[BP]_c = 3.35\,[Hb]_c\,(pH_c - 6.74) \tag{56}$$

For partially oxygenated blood, we substitute $[O_2]_c$ for $[Hb]_c$ in Equation 55, and $[Hb_c] - [O_2]_c$ for $[Hb]_c$ in Equation 56, and add the two equations. We thus obtain for blood with varying degrees of oxygen saturation

$$[BP]_c = 3.35\,[Hb]_c\,(pH_c - 6.74) + [O_2]_c\,0.25\,pH_c - 1.18) \tag{57}$$

It is rather surprising that the buffer value 3.6, in the sense quantitatively defined by Van Slyke (29), found for oxygenated cell contents per mol of hemoglobin is so much greater than the buffer value of recrystallized hemoglobin (previously given as 2.64 (1), but really 3.0 when corrected, as shown in a forthcoming paper, for the inactive hemoglobin present in the preparation). The difference appears, however, to be genuine; for the higher buffer value is also consistent with data we have obtained from hemolyzed blood. The cause may perhaps lie in the fact that in the former experiments the recrystallized hemoglobin was dissolved in solutions containing 30 milli-equivalents of sodium per liter, while in the present solution and in blood the ratio m.-Eq. K per kilo H_2O is about 150. In part the difference may also be due to the presence of slight amounts of non-diffusible cell buffers other than hemoglobin, such as conjugated phosphates.

Saturation Experiments with Blood.

Horse blood was drawn from the jugular vein, and was at once defibrinated and saturated, in portions of 200 cc., with air plus varying amounts of CO_2. The saturations were performed in the 800 cc. saturators illustrated in Fig. 3 of the first paper of this series (5). Our saturators differed slightly in form from the latter, in that, as viewed in longitudinal section, the walls at each end of ours sloped toward the cock at an angle of 60°, in order to facilitate drainage, instead of approaching it in a curve. (This improvement was introduced by Cullen.) The saturations were performed at barometric pressure, and the final CO_2 tension was determined by analysis in a manner described below.

Certain preliminary determinations, indicated in the tables, to ascertain some of the blood constants, were performed on a portion of the defibrinated blood which was not saturated.

The saturated portions were all centrifuged under paraffin, as detailed below. The paraffin protection from the air obviated errors from two sources, either of which would have been of sufficient magnitude to invalidate part of the results. It prevented escape of CO_2, as already shown (5), and it prevented concentration of the serum by evaporation. Centrifugation was continued for about an hour at a speed of 1,500 revolutions per minute, and the cells were obtained so nearly free from serum that no visible layer of the latter separated at the top when a sample of the cells was allowed to stand overnight.

The amounts of blood used were such as to obviate the necessity of using micro methods of analysis, and it was our intention further to insure the certainty of our results by obtaining all the cell data directly from analyses performed on the cells, rather than indirectly from analysis of the serum and whole blood. We succeeded in carrying out this intention in all the determinations except that of chloride. We were not able to satisfy ourselves that the chloride method devised by Austin and Van Slyke (30) for whole blood is entirely accurate for the concentrated cells. The picric acid filtrate from the latter contains something which retards the precipitation of silver chloride, and we did not have time at our disposal to devise a new technique suited to cells. We have accordingly estimated our cell

chloride content by difference from analyses of the whole blood and serum. In consequence we cannot attribute the same degree of accuracy to the cell chloride values as to the bicarbonate values. However, by using relatively large amounts of filtrate (equivalent to 5 cc. of serum or blood) for the titrations, very consistent dupli-

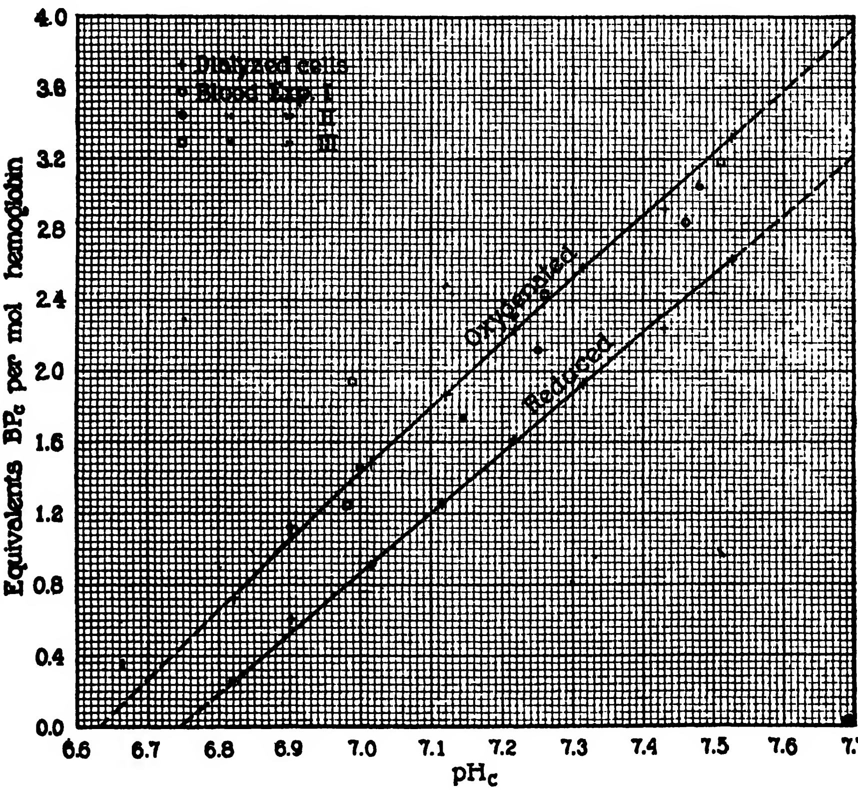

FIG. 10. Equivalents of base bound by reduced and (calculated) oxygenated, non-diffusible cell constituents, per mol of oxygen-combining capacity.

cates were obtained; and we believe the cell chloride figures indicate the changes in distribution resulting from changes in CO_2 tension, although the absolute $[Cl]_c$ values determined may parallel the true values at a distance appreciably below or above the latter.

For the determination of the water shift between serum and cells we abandoned the hematocrit method; the difficulties which it offers

in measuring small changes are familiar. In its place we utilized two independent methods which served as checks on each other; *viz.*, the gravimetric determination of the water content of serum and cells, and specific gravity determination in the serum. The manner of calculating water contents from the results by each of the two procedures is indicated below in the section on "Calculations." Of the two methods it appears that the specific gravity is capable of indicating changes with the higher degree of accuracy. The gravimetric method gave fairly consistent results with cells, but in serum, where the relative water changes were smaller, it does not appear sensitive enough.

The gravities were determined in 50 cc. bottles; the difference between duplicate weighings appears not to exceed 0.5 mg., or 1 part per 100,000. Our water bath during the determinations never varied more than 0.01° in temperature, and usually not over 0.003°. The weight of water held by each bottle was redetermined immediately after each serum determination. It is probable that the ratio $\frac{\text{weight of serum at } 38^\circ}{\text{weight of water at } 38^\circ}$ has an accuracy not much below 1 part per 100,000 and certainly not below 1 per 10,000. An error of the latter magnitude would cause one of about 1 part in 300 in estimating the water: solids ratio in serum.

In the water determinations by both methods the absolute values of $(H_2O)_s$ and $(H_2O)_c$ depend on preliminary cell volume and other determinations and are consequently subject to considerably greater error. The *changes*, however, due to water shift with varying CO_2 tension, are determined with an accuracy dependent only on that of the direct gravimetric or specific gravity estimations.

Saturation Technique.—200 cc. portions of defibrinated horse blood were drawn into evacuated saturators of about 800 cc. capacity. These were filled with the desired mixture of CO_2 and air by the same technique employed in the other experiments, described above. To prevent development of negative pressure the saturators with high CO_2 tensions were rotated at room temperature for a few minutes to hasten the absorption of CO_2, and more air was admitted to restore the pressure before they were placed in the bath. The saturators were then rotated in a bath at 38° until equilibrium was reached.

At intervals during saturation one cock of the saturator was opened to release the pressure. This was repeated until the internal pressure remained equal to that of the atmosphere.

The saturator was finally connected to a 250 cc. centrifuge bottle filled with paraffin oil and provided with a rubber stopper carrying two tubes as shown in Fig. 11*a*. With the saturator still in the bath and almost in a vertical position the blood was transferred to the centrifuge bottle by lowering the leveling bulb connected with the bottle and opening both cocks of the saturator so that air could flow above as blood was drawn out below. Care was taken to see that the rubber and glass tubings connecting the saturator and the bottle had been completely filled with paraffin oil before the saturator cocks were opened.

When all the blood had left the saturator both cocks were closed and the rubber tubings were clamped. The saturator and the bottle were then removed from the bath and disconnected. The rubber stopper was removed from the bottle and the paraffin oil was removed as completely as possible by means of a pipette. Melted paraffin at a temperature of 50° was poured into the bottle, in sufficient amount to form a layer about 1 inch thick on top of the blood. When the paraffin had solidified the blood was centrifuged.

To remove the serum a hole about 1 cm. in diameter was bored through the paraffin by means of a heated cork borer. About 15 cc. of the serum for CO_2 determination were transferred over mercury to a receiver by means of a bent capillary tube,[8] arranged as shown in Fig. 11*b*. As receivers in these experiments we employed the tubes forming the lower chambers of the 2-chambered tonometers described in Paper I (5). Of the remainder of the serum, as much as could be withdrawn without admixture of any cells was transferred to a flask for other analyses by means of an arrangement shown in Fig. 11*c*.

The serum still left on the top of the cells was removed by means of a pipette as completely as possible. To take a sample of cells for CO_2 determination, a rubber stopper carrying a 10 cc. pipette and a bent tube (Fig. 11*d*) was inserted and the cells were forced up into the pipette by gentle air pressure exerted through the bent tube.

[8] See Austin, Cullen, Hastings, McLean, Peters, and Van Slyke (5), p. 132.

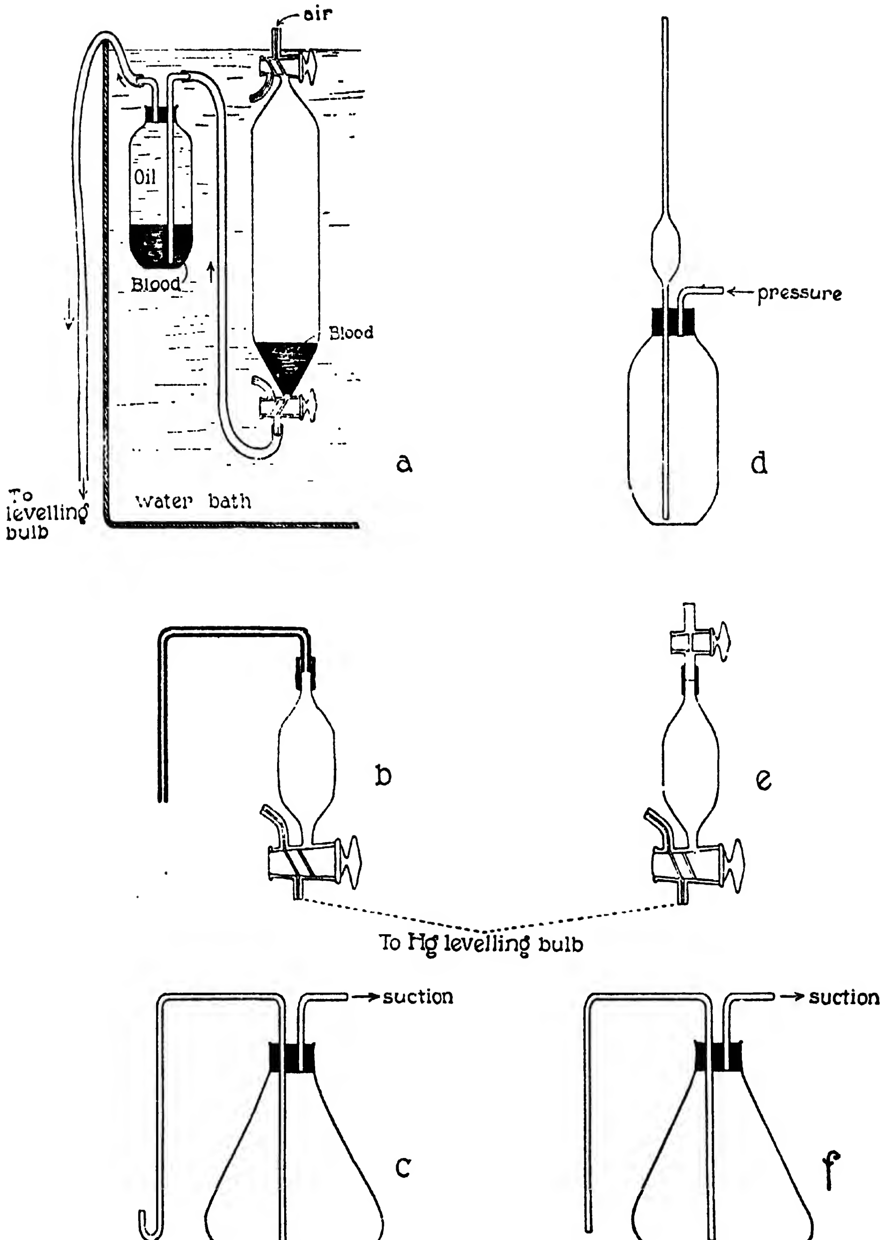

FIG. 11. Apparatus used for handling blood.

The pipette was removed with the stopper, and the cells were delivered under 10 cc. of CO_2-free fluoride-saponin solution, in a receiver of the kind shown in Figs. 11*b* and 11*e*. (The solution contained 0.5 per cent NaF to reduce oxidizing activity and 0.1 per cent saponin to lake the cells and yield a homogeneous solution for analysis.) The receiver plus the solution was weighed before and after the addition of the cells. Finally water was added up to a mark on the neck of the receiver at which its volume capacity had been measured. (It was slightly over 23 cc. for the tubes we used as receivers.) A mercury reservoir was then connected to the bottom cock of the tube and the top was closed by the cock shown in Fig. 11*e*. With the upper cock open mercury was admitted till all the air below this cock was expelled. The solution of the cells was then mixed and allowed to stand a short time before analysis.

The remainder of the cells was transferred for other analyses to a flask by means of an arrangement shown in Fig. 11*f*.

Analytical Methods (Including a Method for Determination of the Total Base in Blood).

The *specific gravity* of the serum at 38° was determined by means of 50 specific gravity bottles as previously described. The specific gravities of the whole blood and serum at room temperature (24°) were determined by means of a 10 cc. pycnometer.

The *dry residue* of the serum and cells was determined by drying at 120° for 18 hours.

The *total* CO_2 contents of the serum and cells were determined by means of the "constant volume" apparatus (31). The apparatus used had a total volume of 50 cc. The gas pressure was measured at 2 cc. volume. 2 cc. of the serum and 3 cc. of the cell solution prepared as described above were used for each CO_2 determination. Each sample was forced into the 2 or 3 cc. pipette by pressure (see "Sampling blood," p. 131 of first paper (5)), and was delivered under a layer of water in the cup of the gas analysis apparatus. The total volume of sample plus water plus 0.5 cc. of N lactic acid was 5 cc. in each case.

The *CO_2 in the saturator gas* was determined by Haldane's method. For calculating the CO_2 tension the percentage found was multiplied by $\frac{v}{v - 200}$ (v = volume of saturator), since the original $v - 200$ cc. of gas mixture in equilibrium with 200 cc. of the blood was diluted by 200 cc. of air when the blood was transferred to the centrifuge bottle. Control experiments in which mercury replaced the blood showed that this procedure was accurate.

The *chloride* of the whole blood was determined in a preliminary sample, using the method of Austin and Van Slyke (30). The chloride of the serum was determined directly, that of the cells was calculated by difference.

The *total base in the serum and in the cells* was determined as follows by a method depending on the principle of Fiske's method (32) for the total base in urine:

About 50 gm. of cells or serum were weighed, and were washed into a 500 cc. flask with 350 cc. of water. In the case of the cells, 50 gm. of trichloroacetic acid, dissolved in 50 cc. of water, were added with constant shaking. The mixture was made up to 500 cc. volume and mixed. For the serum only 15 gm. of trichloroacetic acid were used.

100 cc. of the filtrate were measured into a platinum dish and 2 cc. of concentrated sulfuric acid were added. The mixture was concentrated on a water bath, then the sulfuric acid was driven off with a small flame, and finally the residue was carefully ignited until the ash was white, care being taken that all parts of the dish had been momentarily subjected to a dull red heat.

When the dish had cooled 20 cc. of water and 1 drop of methyl orange were added. The dish was gently agitated until the residue, all or nearly all, had dissolved. The solution was usually neutral. If it was acid it was titrated to neutrality with 0.1 N NaOH from a micro burette. It appears preferable to underheat the sulfates during ignition, and if necessary make a correction for the slight amount of acid left, rather than to risk overheating them and the chance of some loss of SO_3.

0.5 cc. of concentrated HCl was added. The residue (calcium phosphate) dissolved completely. The solution was rinsed into a 200 cc. beaker with 80 cc. of water, and was heated to boiling. 5 cc. of 5 per cent barium chloride solution were added drop by drop. After standing 20 minutes the precipitate was filtered on a Gooch crucible, dried, and weighed.

The barium sulfate weighed represents the total base plus any free sulfuric acid not driven off during the ignition and minus the

base bound by the total phosphoric acid present in the trichloroacetic acid filtrate. The amount of free sulfuric acid left (usually none) was given by the titration. The total phosphoric acid was determined by Brigg's modification (33) of the Bell-Doisy method.

1 molecule of phosphoric acid was taken as equal to one equivalent of base, since the phosphoric acid must be in the form of *m*-phosphate in the ignited residue. The following experiment may be cited to illustrate the method of calculation and the degree of accuracy attainable with the method.

10 cc. of M/10 KCl and 1 cc. of M/30 Na_2HPO_4 were measured into a platinum crucible. 0.5 cc. of H_2SO_4 was added and the mixture was evaporated to dryness and ignited. The residue was dissolved in 50 cc. of water and 1 drop of methyl orange was added. The solution was acid, and 0.78 cc. of 0.1 N NaOH was required for neutralization. The titrated solution was washed into a beaker and the sulfate was determined as described.

0.1296 gm. of $BaSO_4$ corresponding to 0.001109 equivalent of base was obtained. To obtain the total base in the original mixture 0.000033 equivalent due to the phosphate was added to it, and 0.000078 equivalent due to free sulfuric acid was subtracted from it, giving 0.001064 as against the theoretical value of 0.001067 equivalent.

The corrections due to phosphates were found to be 0.002 and 0.003 equivalents per kilo of serum and cells respectively. As these corrections were small in proportion to the total base in the serum and the cells, and varied but little, they were assumed to be constant, and the same values were used for all the determinations.

Methods of Calculation.

The following symbols are used:

mM.	= millimols.
m.-Eq.	= milli-equivalents.
G_b, G_s, G_c,	= specific gravities of blood, serum, and cells at 38°, water at the same temperature being 1.
W_b, W_s, W_c,	= weights of blood, serum, and cells in kilos.
H_2O_b, H_2O_s, H_2O_c,	= water of blood, serum, and cells in kilos.
R_b, R_s, R_c,	= dry residue of blood, serum, and cells in kilos.
P_b, P_s, P_c,	= protein of blood, serum, and cells in grams.
B_b, B_s, B_c,	= base of blood, serum, and cells in milligram equivalents.

Cl, CO_2, H_2CO_3, $BHCO_3$, H^+, similarly to B.

$$[Cl]_s = \frac{Cl_s}{H_2O_s},\ [B]_s = \frac{B_s}{H_2O_s}\ \ldots\ldots \text{ etc.}$$

$$(Cl)_s = \frac{Cl_s}{W_b},\ (B)_s = \frac{B_s}{W_b},\ (B)_c = \frac{B_c}{W_b}\ \ldots\ldots \text{ etc.} = \text{milli-equivalents per kilo blood.}$$

Hb = hemoglobin in milligram molecules of oxygen capacity.

The manner in which our data were calculated is shown by the following formulas, which we used. Equations III to X inclusive indicate the calculation of the blood constants. The specific gravity and volume data of Equations I and II, which are used in the other calculations (except Equation VII) depend on the cell volume, and are therefore constant for a given blood only at a given CO_2 and O_2 tension. Consequently we have made a practice of performing the preliminary Kjeldahl, dry residue, and specific gravity determinations on the cells and serum separated in the course of the cell volume determination, as the simplest way to insure separation of cells and plasma for volume determination and for determination of the blood constants under identical conditions.

It will be noted that all concentrations are calculated as $\dfrac{\text{weight of solute}}{\text{weight of water}}$ or $\dfrac{\text{mols of solute}}{\text{weight of water}}$, in accordance with the basic law concerning osmotic pressure discussed under III in the introduction to this paper.

(I) $$\frac{W_s}{W_b} = \frac{G_s\ V_s}{G_b\ V_b} \quad \text{(from the principle that mass = volume} \times \text{density.)}$$

$\dfrac{V_s}{V_b}$ from centrifugation in graduated tubes.

(II) $$\frac{W_c}{W_b} = 1 - \frac{W_s}{W_b}$$

(III) $$\frac{R_s}{W_b} = \frac{W_s}{W_b} \times \frac{R_s}{W_s}$$

$\dfrac{W_s}{W_b}$ is found from I, $\dfrac{R_s}{W_s}$ from dry residue determination.

(IV) $$\frac{R_c}{W_b} = \frac{W_c}{W_b} \times \frac{R_c}{W_c}$$

$\frac{W_c}{W_b}$ is found from II, $\frac{R_c}{W_c}$ from dry residue determination.

(V)
$$\frac{P_s}{W_b} = \frac{W_s}{W_b} \times \frac{P_s}{W_s}$$

$\frac{W_s}{W_b}$ is found from I, $\frac{P_s}{W_s}$ by Kjeldahl on weighed serum sample.

Protein is calculated as N × 6.25.

(VI)
$$\frac{P_c}{W_b} = \frac{W_c}{W_b} \times \frac{P_c}{W_c}$$

$\frac{W_c}{W_b}$ is found from II, $\frac{P_c}{W_c}$ by Kjeldahl on weighed cell sample.

Cell protein is calculated as N × 5.78 since Kossel (34) gives 17.31 as the N content of horse hemoglobin, and $\frac{1}{0.1731} = 5.78$.

(VII)
$$\frac{Hb}{W_b} = \frac{Hb}{V_b} \times \frac{V_b}{W_b} = \frac{Hb}{V_b \times G_b}$$

$\frac{Hb}{V_b}$ is found by oxygen capacity determination.

(VIII)
$$(B)_s = \frac{B_s}{W_b} = \frac{W_s}{W_b} \times \frac{B_s}{W_s}$$

$\frac{W_s}{W_b}$ is found from I, $\frac{B_s}{W_s}$ by analysis.

(IX)
$$(B)_c = \frac{B_c}{W_b} = \frac{W_c}{W_b} \times \frac{B_c}{W_c}$$

$\frac{W_c}{W_b}$ is found from II, $\frac{B_c}{W_c}$ by analysis of weighed sample of cells.

(X) *f = factor for calculating $\frac{H_2O_s}{W_b}$ from specific gravity of serum.*

For changes as small as those that occur in the solid content of the serum from the water exchange caused by varying CO_2 and O_2 tensions (migration of constituents other than water is a negligible factor), the solid content of the serum may be assumed to vary in a linear manner with $G_s - 1$.

$$\frac{R_s}{W_s} = f\,(G_s - 1)$$

Solving for f, we have

$$f = \frac{R_s}{W_s} \times \frac{1}{G_s - 1}$$

f being determined from G_s and $\frac{R_s}{W_s}$ in a sample of serum separated at any CO_2 tension, we may use f to determine $(H_2O)_s$ in any other serum sample from the same blood. If we substitute $(R)_s + (H_2O)_s$ for W_s in the above and solve for $(H_2O)_s$, we obtain

$$(H_2O)_s = R_s \left(\frac{1}{f\,(G_s - 1)} - 1\right), \text{ or } \frac{(H_2O)_s}{W_b} = \frac{R_s}{W_b}\left(\frac{1}{f\,(G_s - 1)} - 1\right)$$

(XI) $$[H_2CO_3]_s = [H_2CO_3]_c = 0.0324\ P_{CO_2}$$

(Discussion below.)

(XII) $$[CO_2]_s = \frac{CO_{2s}}{H_2O_s} = \frac{W_s}{H_2O_s} \times \frac{CO_{2s}}{W_s} = \frac{W_s}{H_2O_s} \times \frac{CO_{2s}}{V_s\ G_s}$$

$\frac{W_s}{H_2O_s}$ is determined on the weighed serum sample, $\frac{CO_{2s}}{V_s}$ is determined on the measured 2 cc. sample.

Similarly

$$[Cl]_s = \frac{W_s}{H_2O_s} \times \frac{Cl_s}{W_s}$$

$$[B]_s = \frac{W_s}{H_2O_s} \times \frac{B_s}{W_s}$$

and likewise for $[CO_2]_c$, $[Cl]_c$, and $[B]_c$

(XIII) $$[BHCO_3]_s = [CO_2]_s - [H_2CO_3]_s$$

$$[BHCO_3]_c = [CO_2]_c - [H_2CO_3]_c$$

(XIV a) $$(H_2O)_s = \frac{H_2O_s}{W_b} = \frac{H_2O_s}{R_s} \times \frac{R_s}{W_b}$$ from gravimetric water determination.

(XIV b) $$= \frac{R_s}{W_b}\left(\frac{1}{f\,(G_s - 1)} - 1\right)$$ from specific gravity.

$\frac{R_s}{W_b}$ is found from Equation III, f from Equation X.

(XV a) $$(H_2O)_c = \frac{H_2O_c}{W_b} = \frac{H_2O_c}{R_c} \times \frac{R_c}{W_b}$$ from gravimetric water determination.

(XV b) $$= \frac{H_2O_b}{W_b} - \frac{H_2O_s}{W_b}$$ from specific gravity of serum and water content of whole blood.

$\frac{R_c}{W_b}$ is found from Equation IV, $\frac{H_2O_b}{W_b}$ from water determination on whole blood.

$\frac{H_2O_s}{W_b}$ is found from XIV b.

(XVI) $$[Hb]_c = \frac{Hb}{H_2O_c} = \frac{Hb}{W_b} \times \frac{W_b}{H_2O_c}$$

$\frac{Hb}{W_b}$ is found from VII, $\frac{W_b}{H_2O_c}$ from XV.

(XVII) $$[P]_s = \frac{P_s}{H_2O_s} = \frac{P_s}{W_b} \times \frac{W_b}{H_2O_s}$$

$\frac{P_s}{W_b}$ is found from V, $\frac{W_b}{H_2O_s}$ from XIV.

(XVIII) $$[P]_c = \frac{P_c}{H_2O_c} = \frac{P_c}{W_b} \times \frac{W_b}{H_2O_c}$$

$\frac{P_c}{W_b}$ is found from VI, $\frac{W_b}{H_2O_c}$ from XV.

(XIX) $$[H^+] = 7.6 \times 10^{-7} \times \frac{[H_2CO_3]}{[BHCO_3]}$$ (In either cells or serum. Discussion of 7.6 factor below.)

(XX) $$pH = 6.12 + \log \frac{[BHCO_3]}{[H_2CO_3]}$$ (In either cells or serum. Discussion of 6.12 factor below.)

(XXI) $[BP]_s = 0.068\ [P]_s\ (pH_s - 4.80)$

$[P]_s$ from XVII. Numerical constants from Equation 54.

(XXII) $[BP]_c = [Hb]_c \times$ (Equivalents base bound per mol Hb.)

The base bound per mol was estimated graphically from Fig. 10.

$[Hb]_c$ from XVI.

Discussion of Methods of Calculation.—For the most part the formulas used are obvious, but the following demand some explanation.

Equation VII. The Millimolar Concentration of Hemoglobin.—The millimolar concentration of hemoglobin per 1,000 gm. of blood was estimated from the oxygen capacity on the assumption that 1 molecule of hemoglobin binds 1 of oxygen when saturated with atmospheric air.

The grams of hemoglobin are estimated by multiplying the millimolar oxygen capacity of the blood by 16.7. Hüfner showed that 1 molecule of oxygen or carbon monoxide combines with 16,700 gm. of ox hemoglobin, and for lack of figures determined directly on horse hemoglobin we have used this factor. The ***hemoglobin weights*** that we give can be considered as only approximately accurate, because of the uncertainty regarding the degree of exactness of the factor 16.7. We have, however, indicated the weights calculated by it, in order to check the relationships between hemoglobin, total cell protein, and cell solids.

Equation XI. $[H_2CO_3]$, *Milligram Molecules of Free* CO_2 *per 1,000 Gm.* H_2O *in Cells or Serum.*—$[H_2CO_3]$ has been calculated on the assumption that in the water of both cells and serum CO_2 has the same solubility as in a salt solution with the same salt: H_2O ratio, *viz.* 160 mM. of salt per 1,000 gm. of water. Geffcken (35) has found that presence of an inorganic colloid (As_2S_3) does not alter the solvent power of water for CO_2. It appears that the proteins present in blood behave in a similar manner, affecting the solvent power of the water as little as they do its vapor tension (Neuhausen, 7).

From the data on the relative solubility of CO_2 in KCl solutions (Geffcken) and in NaCl solutions (Bohr, quoted by Geffcken) the mean solubility of CO_2 per gram of water in 0.16 M solutions of these salts is estimated at 98.8 per cent of its solubility in pure water.

Estimation of α_{CO_2} at 38° in the blood water as 98.8 per cent of that in pure water gives $\alpha_{CO_2} = 0.988 \times 0.555 = 0.548$; whence mM. of H_2CO_3 per *liter* of water in blood $= \dfrac{0.548\ p_{CO_2}}{2.24 \times 760} = 0.0322\ p_{CO_2}$. To calculate H_2CO_3 per *kilo* of blood water, we multiply the factor 0.0322 by 1.007, the volume in liters of a kilo of water at 38°. Hence $[H_2CO_3] = 0.0324\ p_{CO_2}$.

If, on the basis of the above assumption that the blood colloids do not significantly alter the solubility of CO_2 in the blood water, we calculate the *relative* solubility coefficient (the solubility per unit volume of solution compared with the solubility per unit volume of water) for CO_2 in the total serum we obtain the value $0.988 \times 0.92 \times 1.027 = 0.935$. (0.988 = relative solubility in serum water; 0.92 = gm. of water per gm. of serum; 1.027 = specific gravity of serum.)

Similarly, assuming that the cells contain 65 per cent of water and have a specific gravity of 1.10, we calculate that the relative CO_2 solubility coefficient is $0.988 \times 0.65 \times 1.10 = 0.71$.

Bohr (36) found that oxygen had a relative solubility of 0.975 in serum, and assumed the same relative solubility for CO_2. For blood cells Bohr estimated indirectly, from hydrogen solubilities in whole blood, a relative CO_2 solubility of 0.81. Both of Bohr's coefficients are higher than those estimated by us from the H_2O and salt content of serum and cells.

However, the principle of parallel gas solubilities used by Bohr was shown by Geffcken in 1904 (35) to be inexact. "Die Grösse der relativen Löslichkeitsverniedrigung ist sehr abhängig von der Nature des Gases." Bohr's own data on the solubility ratios of O_2 and N_2 in various water solutions show considerable variation. The accuracy with which Bohr's O_2 and H_2 relative solubilities can be translated into CO_2 relative solubilities is according to both Geffcken's results and his own, uncertain.

Some preliminary solubility determinations performed directly with CO_2 itself in serum and cell contents acidified to a pH sufficiently low to prevent the combination of any CO_2 with bases have given results more in accord with the coefficients we have estimated from the water content than with those Bohr has estimated from H_2 and O_2 solubilities. The use of a CO_2 solubility factor proportional to the water content also corrects automatically for variations in the solid content of the cells and serum. While minor factors may be found to alter the CO_2 solubilities calculated as proportional to the water content, the solubilities so calculated appear sufficiently close to serve our present purposes; *e.g.*, the difference between the pH_s calculated by our solubility coefficient and the pH_s calculated by

Bohr's is only 0.02, and the correct coefficient, if it is not exactly the one we use, appears to be nearer to it than to Bohr's. The pH_s values calculated by Equation XX may, therefore, be assumed to be subject to less than 0.01 pH error from possible inaccuracy in α_{CO_2}.

Equation XIX. $[H^+]$, Gram Equivalent of Ionized Hydrogen per 1,000 Gm. of Water.—The value of the K′ of Henderson's equation $[H^+] = K' \frac{[H_2CO_3]}{[BHCO_3]}$, we have calculated for serum from the results of Cullen (28). We have calculated $[H_2CO_3]$ and $[BHCO_3]$ as in Equations XI and XIII above, and estimated pK′, the negative logarithm of K′, as $pK' = pH - \log \frac{[BHCO_3]}{[H_2CO_3]}$. $[CO_2]$ has been estimated as mM. CO_2 per liter $\times$ 1.06 $\left(1.06 = \frac{1}{1.026 \times 0.92}\right.$, 1.026 being taken as the average specific gravity of horse serum, 0.92 as the water content). Cullen's data yield for both serum and plasma of horse blood the average pK′ value of 6.12, the maximum variation (except in one determination out of twenty-three) being from 6.09 to 6.14.

We have therefore used for the constant K′, the value 7.58×10^{-7} of which 6.12 is the negative logarithm.

Warburg (10) has shown that the pK′ value is a function of the concentration of cations, $pK' = pK_1 - 0.46 \sqrt[3]{[B]}$, pK_1 being the value of pK′ in bicarbonate solutions at infinite dilution. $[B]_s$ and $[B]_c$ in blood are so near together, that the extreme difference observed in our experiments would cause pK'_s to differ from pK'_c by only about 0.01. We have therefore used the same K′ and pK′ values for cells as for serum.

Warburg's data from $NaHCO_3$ + NaCl solutions indicate for $[B] = 0.166$ to 0.170 N, values for pK′ of 6.11, 6.08, and 6.11 respectively when reduced from Bjerrum's pH unit to Sörensen's by subtracting 0.05.[9] The agreement with the 6.12 value obtained as the mean of Cullen's results is satisfactory.

[9] See Warburg (10), p. 259, Table XLIV.

Explanation of Tables VIII, IX, X, and XI.—The abbreviations used have already been defined on pp. 410–411. The data from each experiment are given in four sections headed *a*, *b*, *c*, and *d*, of the corresponding table. The calculations involved have already been outlined.

In the final section (headed *d*), the Roman numerals, I, II, III, used at the left of the pages, indicate the laws of solutions involved in the blood relationships demonstrated, the laws being referred to by the numeral headings under which they are respectively discussed in the theoretical introduction of the paper.

In the tabulation of data showing the osmolar relationships of cells and serum the symbol X is used to indicate the small portion of non-protein anions other than Cl′ and HCO_3'; *i.e.*,

$$\Sigma\,[M]_s = [B]_s + [Cl]_s + [HCO_3]_s + [X]_s, \text{ hence } [B]_s + [Cl]_s + [HCO_3]_s = \Sigma\,[M]_s - [X]_s$$

TABLE VIII *a*.

Experiment 1.

Preliminary Determinations for Calculation of Blood Constants.

Determination.	Symbol.	Unit.	Blood.	Serum.	Cell.
Oxygen capacity per l.	$Hb:V_b$	mM.	5.69		
Volume per l.	$V_s:V_b$, $V_c:V_b$	l.		0.710	0.290
Water per gm.	$H_2O_b:W_b$, $H_2O_s:W_s$, $H_2O_c:W_c$	gm.	0.8357	0.9188	0.6390
Solids per gm.	$R_b:W_b$, $R_s:W_s$, $R_c:W_c$	"	0.1643	0.0812	0.3610
Nitrogen per gm.	$N_s:W_s$, $N_c:W_c$	"		0.0118	0.0579
Protein per gm.	$P_s:W_s$, $P_c:W_c$	"		0.0738*	0.3342†
Chloride per kilo.	$Cl_b:W_b$	m.-Eq.	82.7		
Base per kilo.	$B_s:W_s$, $B_c:W_c$	"			
Specific gravity $\frac{24°}{24°}$	G_b, G_s		1.0457	1.0257	
Weight per gm. blood	$W_s:W_b$, $W_c:W_b$	gm.		0.697	0.303

*N × 6.25.
†N × 5.78.

TABLE VIII *b*.

Experiment 1.

Calculated Constants.

Constants.	Symbols.	Unit.	
Hemoglobin per kilo blood	(Hb)	mM.	5.43
" " gm. "		gm.	0.0907
Total solids " " "	$(R)_b = R_b:W_b$	"	0.1643
Serum " " " "	$(R)_s = R_s:W_b$	"	0.0557
Cell " " " "	$(R)_c = R_c:W_b$	"	0.1072
Serum protein " " "	$(P)_s = P_s:W_b$	"	0.0500
Cell " " " "	$(P)_c = P_c:W_b$	"	0.0990
Serum base " kilo "	$(B)_s = B_s:W_b$	mM.	99.8
Cell " " " "	$(B)_c = B_c:W_b$	"	34.2
Factor for calculated $H_2O_s:W_s$ from $G_s \frac{38°}{38°}$	f		3.09*

* From Table VIII *c*.

TABLE VIII *c*.

Experiment 1.

Determinations after Saturations at 38°.

Determination.	Symbol.	Unit.	Blood 1.		Blood 2.	
			Serum.	Cells.	Serum.	Cells.
CO_2 tension	p_{CO_2}	mm. Hg	19.3	19.3	106.7	106.7
CO_2 per kilo	$CO_{2s}:W_s$, $CO_{2c}:W_c$	mM.	20.38	9.29	34.18	19.53
Cl " "	$Cl_s:W_s$, $Cl_c:W_c$	m.-Eq.	100.2	41.8	95.9	53.0
Base " "	$B_s:W_s$, $B_c:W_c$	"	145.3	109.2	148.1	108.6
Water per gm.	$H_2O_s:W_s$, $H_2O_c:W_c$	gm.	0.9186	0.6508	0.9190	0.6665
Solids " "	$R_s:W_s$, $R_c:W_c$	"	0.0814	0.3494	0.0810	0.3335
Ratio, water : solids	$H_2O_s:R_s$, $H_2O_c:R_c$		11.28	1.864	11.35	1.998
Specific gravity $\frac{38°}{38°}$	G_s		1.02595		1.02660	

TABLE VIII *d.*

Experiment 1.

Values Calculated from Determinations on Blood Saturated at 38°.

Relationship demonstrated.	Values calculated	Symbol and equation.	Unit.	Blood 1.		Blood 2.	
				Serum.	Cells.	Serum.	Cells.
	CO_2 per kg. water.	$[CO_2]$	mM.	22.16	14.27	37.18	29.30
	H_2CO_3 " " "	$[H_2CO_3]$	"	0.625	0.625	3.46	3.46
	$BHCO_3$ " " "	$[BHCO_3]$	"	21.52	13.64	33.72	25.84
	Cl " " "	[Cl]	m.-Eq.	109.1	64.2	104.3	79.5
	Base " " "	[B]	"	158.1	167.6	161.1	162.8
	" " " blood.	(B)	"	99.4	33.5	100.2	34.8
	Hemoglobin per kg. water.	[Hb]	mM.		27.0		25.4
	Protein per kg. water.	[P]	gm.	79.5	487	80.9	463
	pH	pH	$-\log [H^+]$	7.658	7.459	7.109	6.993
	Base bound by protein per kg. water.	[BP]	m.-Eq.	15.7	83.5	12.7	35.1
Equality of ratio $\frac{\text{ions + mols}}{\text{water}}$ in serum and cells. III	Total osmolar concentration − X. Observed.	$\Sigma [M] - X = [B] + [Cl] + [HCO_3] + [Hb]$, Equation 5.	mM.	289	272	299	294
	Total osmolar concentration. Calculated.	$\Sigma [M] = 2[B] - [BP] + [Hb]$, Equation 7.	"	300	279	310	316

Water distribution. III	Water per kg. blood. Observed gravimetric.	(H_2O)	kg.	0.629	0.200		0.214
	Water per kg. blood. Observed sp. gr.	"	"	0.639	0.197	0.622	0.214
	Water per kg. blood. Calculated.	" by Equations 23 and 24.	"	0.643	0.193	0.621	0.215
	Water shift from Blood 1. Observed gravimetric.	$\Delta(H_2O)$	"	0.000	0.000		0.014
	Water shift from Blood 1. Observed sp. gr.	"	"	0.000	0.000	0.017	0.017
	Water shift from Blood 1. Calculated.	" by Equations 23 and 24.	"	0.000	0.000	0.022	0.022
Diffusible ion distribution. I, II, III	Distribution ratio. Observed.	$[Cl]_c : [Cl]_s$	Ratio.	0.589		0.762	
		$[HCO_3]_c : [HCO_3]_s = [H^+]_s : [H^+]_c$	"	0.634		0.767	
	Distribution ratio. Calculated.	r by Equation 10.	"	0.667		0.846	
		" " " 14.	"	0.667		0.853	

TABLE IX *a.*

Experiment 2.

Preliminary Determinations for Calculation of Blood Constants.

Determination.	Symbol.	Unit.	Blood.	Serum.	Cell.
Oxygen capacity per l.	$Hb:V_b$	mM.	8.02		
Volume per l.	$V_s:V_b$, $V_c:V_b$	l.		0.618	0.382
Water per gm.	$H_2O_b:W_b$, $H_2O_s:W_s$, $H_2O_c:W_c$	gm.	0.8093	0.9136	0.6327
Solids per gm.	$R_b:W_b$, $R_s:W_s$, $R_c:W_c$	"	0.1907	0.0864	0.3673
Nitrogen per gm.	$N_s:W_s$, $N_c:W_c$	"		0.01135	0.05410
Protein per gm.	$P_s:W_s$, $P_c:W_c$	"		0.0709*	0.3127†
Chloride per kilo.	$Cl_b:W_b$	m.-Eq.	83.2		
Base per kilo.	$B_s:W_s$, $B_c:W_c$	"			
Specific gravity $\frac{24°}{24°}$	G_b, G_s		1.0548	1.0282	
Weight per kilo.	$W_s:W_b = G_sV_s:G_bV_b$, $W_c:W_b = 1\text{-}W_s:W_b$			0.602	0.398
Specific gravity $\frac{38°}{38°}$	G_s			1.027859	

* N × 6.25.

† N × 5.78.

TABLE IX *b*.

Experiment 2.

Calculated Constants.

Constants.	Symbols.	Unit.	
Hemoglobin per kilo blood	(Hb)	mM.	7.61
" " gm. "		gm.	0.1272
Total solids " " "	$(R)_b = R_b:W_b$	"	0.1907
Serum " " " "	$(R)_s = R_s:W_b$	"	0.0521
Cell " " " "	$(R)_c = R_c:W_b$	"	0.1461
Serum protein " " "	$(P)_s = P_s:W_b$	"	0.0427
Cell " " " "	$(P)_c = P_c:W_b$	"	0.1243
Serum base " kilo "	$(B)_s = B_s:W_b$	mM.	90.0*
Cell " " " "	$(B)_c = B_c:W_b$	"	43.0*
Factor for calculating $H_2O_s:W_s$ from $G_s \frac{38°}{38°}$	f		3.102

* Mean of determinations on Bloods 1, 2, 3, and 4.

TABLE IX*c*—

Determinations after

Determination.	Symbol.	Unit.
CO_2 tension	P_{CO_2}	mm. Hg
CO_2 per kilo	$CO_{2s}:W_s$, $CO_{2c}:W_c$	mM.
Cl " "	$Cl_s:W_s$, $Cl_c:W_c$	m.-Eq.
Base " "	$B_s:W_s$, $B_c:W_c$	"
Water " gm.	$H_2O_s:W_s$, $H_2O_c:W_c$	gm.
Solids " "	$R_s:W_s$, $R_c:W_c$	"
Ratio, water: solids	$H_2O_s:R_s$, $H_2O_c:R_c$	
Specific gravity $\frac{38°}{38°}$	G_s	

TABLE IX*d*—

Values Calculated from Determi-

Relationship demonstrated.	Values calculated.	Symbol and equation.
	CO_2 per kg. water.	$[CO_2]$
	H_2CO_3 " " "	$[H_2CO_3]$
	$BHCO_3$ " " "	$[BHCO_3]$
	Cl " " "	[Cl]
	Base " " "	[B]
	" " " blood.	(B)
	Hemoglobin per kg. water.	[Hb]
	Protein " " "	[P]
	pH	pH
	Base bound by protein per kg. water.	[BP]
Equality of ratio $\frac{\text{ions + mols}}{\text{water}}$ in serum and cells. III	Total osmolar concentration − X. Observed.	$\Sigma[M] - X = [B] + [Cl] + [HCO_3] + [Hb]$, Equation 5.
	Total osmolar concentration. Calculated.	$\Sigma[M] = 2[B] - [BP] + [Hb]$, Equation 7.
Water distribution. III	Water per kg. blood. Observed gravimetric.	(H_2O)
	Water per kg. blood. Observed sp. gr.	"
	Water per kg. blood. Calculated.	" by Equations 23 and 24.
	Water shift from Blood 1. Observed gravimetric.	$\Delta(H_2O)$
	Water shift from Blood 1. Observed sp. gr.	"
	Water shift from Blood 1. Calculated.	" by Equations 23 and 24.
Diffusible ion distribution. I, II, III	Distribution ratio. Observed.	$[Cl]_c:[Cl]_s$ $[HCO_3]_c:[HCO_3]_s = [H^+]_s:[H^+]_c$
	Distribution ratio. Calculated.	r by Equation 10. " " " 14.

Experiment 2.

Saturation at 38°.

Blood 1.		Blood 2.		Blood 3.		Blood 4.	
Serum.	Cells.	Serum.	Cells.	Serum.	Cells.	Serum.	Cells.
16.96	16.96	44.6	44.6	69.3	69.3	118.4	118.4
20.34	10.50	27.63	13.32	31.60	16.56	37.40	21.40
108.1		104.3		103.4		102.0	
143.4	109.2	149.5	104.5	154.3	103.5	154.8	104.5
0.9147	0.6293	0.9138	0.6358	0.9137	0.6377	0.9134	0.6480
0.0853	0.3707	0.0862	0.3642	0.0863	0.3623	0.0866	0.3520
10.73	1.697	10.60	1.745	10.58	1.760	10.55	1.840
1.027434		1.027915		1.028119		1.028235	

Experiment 2.

nations on Blood Saturated at 38°.

Unit.	Blood 1.		Blood 2.		Blood 3.		Blood 4.	
	Serum.	Cells.	Serum.	Cells.	Serum.	Cells.	Serum.	Cells.
mM.	22.22	(13.04)	30.23	20.95	34.58	25.97	40.93	32.70
"	0.550	0.550	1.445	1.445	2.245	2.245	3.83	3.83
"	21.67	(12.49)	28.78	19.50	32.34	23.72	37.72	28.87
m.-Eq.	118.2	68.1	114.2	78.8	113.1	81.8	111.6	85.1
"	156.8	173.7	163.6	164.5	168.8	162.4	169.5	161.3
"	87.9	43.2	89.8	42.8	92.0	42.9	90.0	43.2
mM.		30.5		29.3		28.8		28.3
gm.	76.2	499	77.8	478	78.4	471	78.8	464
$-\log [H^+]$	7.714	(7.476)	7.419	7.250	7.274	7.145	7.106	6.997
m.-Eq.	15.1	96.2	13.9	69.1	13.2	56.6	12.4	39.8
mM.	297	285	307	292	314	297	318	299
"	298	282	313	289	324	297	327	311
kg.	0.559	0.248	0.553	0.255	0.552	0.257	0.550	0.269
"	0.560	0.249	0.550	0.260	0.545	0.264	0.541	0.268
"	0.576	0.233	0.561	0.248	0.555	0.254	0.547	0.262
"	0.000	0.000	0.006	0.007	0.007	0.009	0.009	0.021
"	0.000	0.000	0.011	0.011	0.015	0.015	0.019	0.019
"	0.000	0.000	0.015	0.015	0.021	0.021	0.029	0.029
Ratio.	0.576		0.690		0.723		0.762	
"	—		0.678		0.734		0.779	
"	0.606		0.718		0.768		0.823	
"	0.600		0.706		0.764		0.821	

TABLE X *a.*

Experiment 3.

Preliminary Determinations for Calculation of Blood Constants.

Determination.	Symbol.	Unit.	Blood.	Serum.	Cell.
Oxygen capacity per l.	$Hb:V_b$	mM.	6.62		
Volume per l.	$V_s:V_b$, $V_c:V_b$	l.		0.6775	0.3225
Water per gm.	$H_2O_b:W_b$, $H_2O_s:W_s$, $H_2O_c:W_c$	gm.	0.8206	0.9143	0.6300
Solids per gm.	$R_b:W_b$, $R_s:W_s$, $R_c:W_c$	"	0.1794	0.0857	0.3700
Nitrogen per gm.	$N_s:W_s$, $N_c:W_c$	"		0.01153	0.05715
Protein per gm.	$P_s:W_s$, $P_c:W_c$	"		0.0721*	0.3302†
Chloride per kilo.	$Cl_b:W_b$	m.-Eq.	80.7		
Base per kilo.	$B_s:W_s$, $B_c:W_c$	"			
Specific gravity $\frac{24°}{24°}$	G_b, G_s		1.051	1.027	
Weight per kilo.	$W_s:W_b = G_sV_s:G_bV_b$, $W_c:W_b = 1\text{-}W_s:W_b$			0.662	0.338
Specific gravity $\frac{38°}{38°}$	G_s			1.027332	

* N × 6.25.

† N × 5.78.

TABLE X *b*.

Experiment 3.

Calculated Constants.

Constants.	Symbols.	Unit.	
Hemoglobin per kilo blood	(Hb)	mM.	6.30
" " gm. "		gm.	0.1052
Total solids " " "	$(R)_b = R_b:W_b$	"	0.1794
Serum " " " "	$(R)_s = R_s:W_b$	"	0.0567
Cell " " " "	$(R)_c = R_c:W_b$	"	0.1252
Serum protein " " "	$(P)_s = P_s:W_b$	"	0.0477
Cell " " " "	$(P)_c = P_c:W_b$	"	0.1116
Serum base " kilo "	$(B)_s = B_s:W_b$	m.-Eq.	97.7
Cell " " " "	$(B)_c = B_c:W_b$	"	38.9
Factor for calculating $H_2O_s:W_s$ from $G_s\frac{38°}{38°}$	f		3.135

TABLE X *c*.

Experiment 3.

Determinations after Saturation at 38°.

Determination.	Symbol.	Unit.	Blood 1.		Blood 2.		Blood 3.	
			Serum.	Cells.	Serum.	Cells.	Serum.	Cells.
CO_2 tension	p_{CO_2}	mm. Hg	14.52	14.52	40.9	40.9	117.2	117.2
CO_2 per kilo	$CO_{2s}:W_s$, $CO_{2c}:W_c$	mM.	18.48	7.69	25.12	12.55	35.02	20.50
Cl " "	$Cl_s:W_s$, $Cl_c:W_c$	m.-Eq.	98.60		95.6		92.6	
Base " "	$B_s:W_s$, $B_c:W_c$	"	144.7	113.3	148.4	111.5	153.2	104.3
Water " gm.	$H_2O_s:W_s$, $H_2O_c:W_c$	gm.	0.9158	0.6350	0.9136	0.6415	0.9137	0.6514
Solids " "	$R_s:W_s$, $R_c:W_c$	"	0.0842	0.3650	0.0864	0.3585	0.863	0.3486
Ratio, water : solids	$H_2O_s:R_s$, $H_2O_c:R_c$		10.87	1.740	10.60	1.790	10.58	1.817
Specific gravity $\frac{38°}{38°}$	G_s		1.027256		1.027889		1.028023	

TABLE X *d*—

Values Calculated from Determi-

Relationship demonstrated.	Values calculated.	Symbol and equation.
	CO_2 per kg. water.	$[CO_2]$
	H_2CO_3 " " "	$[H_2CO_3]$
	$BHCO_3$ " " "	$[BHCO_3]$
	Cl " " "	[Cl]
	Base " " "	[B]
	" " " blood.	(B)
	Hemoglobin per kg. water.	[Hb]
	Protein " " "	[P]
	pH	pH
	Base bound by protein per kg. water.	[BP]
Equality of ratio $\frac{\text{ions + mols}}{\text{water}}$ in serum and cells. III	Total osmolar concentration − X. Observed.	$\Sigma [M] - X = [B] + [Cl] + [HCO_3] + [Hb]$, Equation 5.
	Total osmolar concentration. Calculated.	$\Sigma [M] = 2[B] - [BP] + [Hb]$, Equation 7.
Water distribution. III	Water per kg. blood. Observed gravimetric.	(H_2O)
	Water per kg. blood. Observed sp. gr.	"
	Water per kg. blood. Calculated.	" by Equations 23 and 24.
	Water shift from Blood 1. Observed gravimetric.	$\Delta (H_2O)$
	Water shift from Blood 1. Observed sp. gr.	"
	Water shift from Blood 1. Calculated.	" by Equations 23 and 24.
Diffusible ion distribution. I, II, III	Distribution ratio. Observed.	$[Cl]_c:[Cl]_s$
		$[HCO_3]_c:[HCO_3]_s = [H^+]_s:[H^+]_c$
	Distribution ratio. Calculated.	r by Equation 10.
		" " " 14.

Experiment 3.

nations on Blood Saturated at 38°.

Unit.	Blood 1.		Blood 2.		Blood 3.	
	Serum.	Cells.	Serum.	Cells.	Serum.	Cells.
mM.	20.36	12.11	27.50	19.56	38.34	31.48
"	0.470	0.470	1.325	1.325	3.80	3.80
"	19.89	11.64	26.17	18.13	34.54	27.68
m.-Eq.	107.6	66.3	105.7	76.3	101.3	84.8
"	158.0	178.4	162.5	173.8	167.7	162.2
"	97.6	38.9	97.8	39.0	100.6	36.5
mM.		28.9		28.1		27.7
gm.	77.4	521	79.2	497	79.4	491
$-\log [H^+]$	7.747	7.513	7.416	7.257	7.078	6.982
m.-Eq.	15.7	94.9	14.1	66.9	12.3	37.4
mM.	286	285	294	296	303	302
"	300	291	311	309	323	315
kg.	0.616	0.218	0.602	0.224	0.600	0.228
"	0.608	0.213	0.592	0.229	0.589	0.232
"	0.612	0.209	0.600	0.221	0.586	0.235
"	0.000	0.000	0.014	0.006	0.016	0.010
"	0.000	0.000	0.016	0.016	0.019	0.019
"	0.000	0.000	0.012	0.012	0.026	0.026
Ratio.	0.616		0.722		0.837	
"	0.586		0.693		0.802	
"	0.620		0.727		0.830	
"	0.629		0.740		0.845	

TABLE XI a.

Experiment 4.

Preliminary Determinations for Calculation of Blood Constants.

Determination.	Symbol.	Unit.	Blood.	Serum.	Cell.
Oxygen capacity per l.	$Hb:V_b$	mM.	6.91		
Volume per l.	$V_s:V_b$, $V_c:V_b$	l.		0.663	0.337
Water per gm.	$H_2O_b:W_b$, $H_2O_s:W_s$, $H_2O_c:W_c$	gm.	0.8170	0.9103	
Solids per gm.	$R_b:W_b$, $R_s:W_s$, $R_c:W_c$	"	0.1830	0.0897	
Nitrogen per gm.	$N_s:W_s$, $N_c:W_c$	"		0.01126	0.0538
Protein per gm.	$P_s:W_s$, $P_c:W_c$	"		0.0704*	0.312†
Chloride per kilo.	$Cl_b:W_b$	m.-Eq.	77.4		
Base per kilo.	$B_s:W_s$, $B_c:W_c$	"		148.8	120.0
Specific gravity $\frac{24°}{24°}$	G_b, G_s		1.052	1.027	
Weight per kilo.	$W_s:W_b = G_sV_s:G_bV_b$, $W_c:W_b = 1-W_s:W_b$			0.647	0.353
Specific gravity $\frac{38°}{88°}$	G_s			1.028353	

* N × 6.25.

† N × 5.78.

TABLE XI *b*.

Experiment 4.

Calculated Constants.

Constants.	Symbols.	Unit.	
Hemoglobin per kilo blood	(Hb)	mM.	6.57
" " gm. "		gm.	0.1096
Total solids " " "	$(R)_b = R_b:W_b$	"	0.1830
Serum " " " "	$(R)_s = R_s:W_b$	"	0.05804
Cell " " " "	$(R)_c = R_c:W_b$	"	0.1250
Serum protein " " "	$(P)_s = P_s:W_b$	"	0.0455
Cell " " " "	$(P)_c = P_c:W_b$	"	0.1102
Serum base " kilo "	$(B)_s = B_s:W_b$	m.-Eq.	96.3
Cell " " " "	$(B)_c = B_c:W_b$	"	(39.4)*
Factor for calculating $H_2O_s:W_s$ from $G_s \frac{38°}{38°}$	f		3.165

* Calculated as 6(Hb). Direct determination lost.

TABLE XI *c*.

Experiment 4.

Determinations after Saturation at 38°.

Determination.	Symbol.	Unit.	Blood 1.		Blood 2.		Blood 3.	
			Serum.	Cells.	Serum.	Cells.	Serum.	Cells.
CO_2 tension	p_{CO_2}	mm. Hg	19.6	19.6	52.9	52.9	127.3	127.3
CO_2 per kilo	$CO_{2s}:W_s$, $CO_{2c}:W_c$	mM.	21.28	9.13	28.23	15.00	35.90	21.95
Cl " "	$Cl_s:W_s$, $Cl_c:W_c$	m.-Eq.	96.7		94.7		91.6	
Specific gravity $\frac{38°}{38°}$	G_s		1.027943				1.028774	

TABLE XI*d*—

Values Calculated from Determi-

Relationship demonstrated.	Values calculated.	Symbol and equation.
	CO_2 per kg. water. H_2CO_3 " " " $BHCO_3$ " " " Cl " " " Base " " " Hemoglobin per kg. water. Protein " " " pH Base bound by protein per kg. water.	$[CO_2]$ $[H_2CO_3]$ $[BHCO_3]$ [Cl] [B] [Hb] [P] pH [BP]
Equality of ratio $\frac{\text{ions + mols}}{\text{water}}$ in serum and cells. III	Total osmolar concentration − X. Observed. Total osmolar concentration. Calculated.	$\Sigma [M] - X = [B] + [Cl] + [HCO_3] + [Hb]$, Equation 5. $\Sigma [M] = 2[B] - [BP] + [Hb]$, Equation 7.
Water distribution. III	Water per kg. blood. Observed gravimetric. Water per kg. blood. Observed sp. gr. Water per kg. blood. Calculated. Water shift from Blood 1. Observed gravimetric. Water shift from Blood 1. Observed sp. gr. Water shift from Blood 1. Calculated.	(H_2O) " " by Equations 23 and 24. $\Delta (H_2O)$ " " by Equations 23 and 24.
Diffusible ion distribution. I, II, III	Distribution ratio. Observed. Distribution ratio. Calculated.	$[Cl]_c:[Cl]_s$ $[HCO_3]_c:[HCO_3]_s = [H^+]_s:[H^+]_c$ r by Equation 10.

Experiment 4.

nations on Blood Saturated at 38°.

Unit.	Blood 1.		Blood 2.		Blood 3.	
	Serum.	Cells.	Serum.	Cells.	Serum.	Cells.
mM.	23.35	14.35	31.00	22.91	39.55	33.50
"	0.635	0.635	1.715	1.715	4.122	4.122
"	22.71	13.61	29.28	21.19	35.43	29.38
m.-Eq.	106.3	63.5	104.1	70.7	100.8	79.8
"	161.7	(181.5)	163.9	(173.6)	166.3	(165.6)
mM.		30.0		28.7		27.6
gm.	76.1	571	77.4	528	78.6	525
$-\log [H^+]$	7.686	7.450	7.352	7.212	7.055	6.972
m.-Eq.	15.0	90.8	13.4	63.7	12.1	36.2
mM.	290	289	297	294	303	302
"	308	302	314	312	321	323
kg.	—	—	—	—	—	—
"	0.598	0.219	—	—	0.579	0.238
"	0.602	0.215	0.591	0.226	0.579	0.238
"	—	—	—	—	—	—
"	0.000	0.000	—	—	0.019	0.019
"	0.000	0.000	0.011	0.011	0.023	0.023
Ratio.	0.597		0.679		0.792	
"	0.597		0.723		0.830	
"	0.639		0.738		0.832	

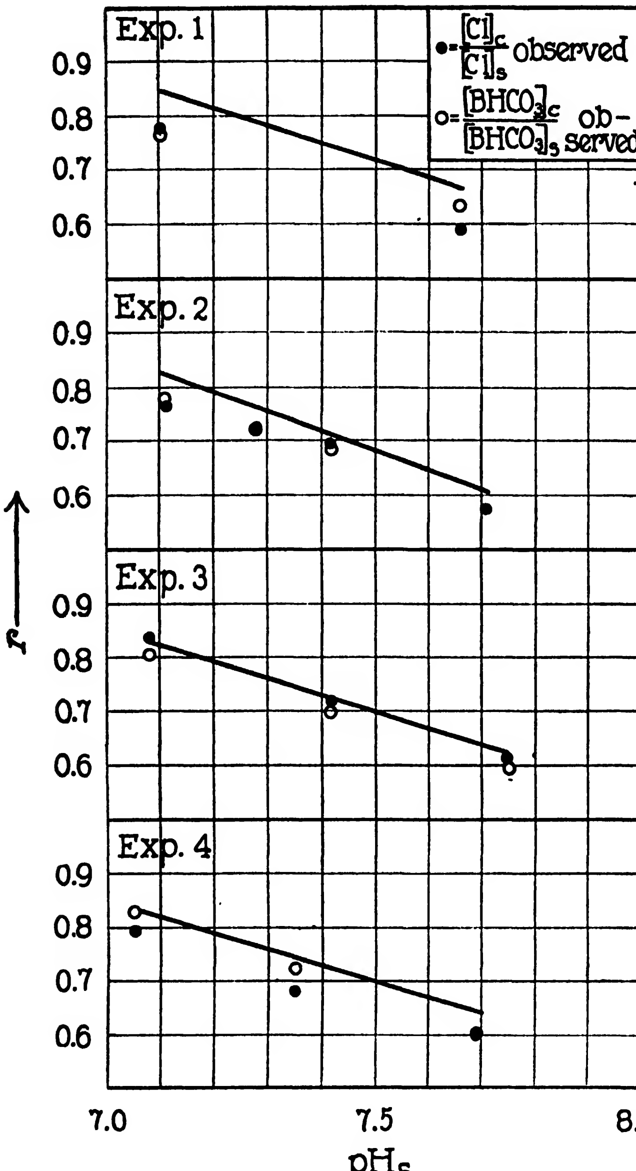

FIG. 12. Comparison of observed $[Cl']_c : [Cl']_s$ and $[BHCO_3']_c : [BHCO_3']_s$ values with values calculated by Equation 10 from base-binding powers of cell and serum proteins. Calculated values are indicated by the curves.

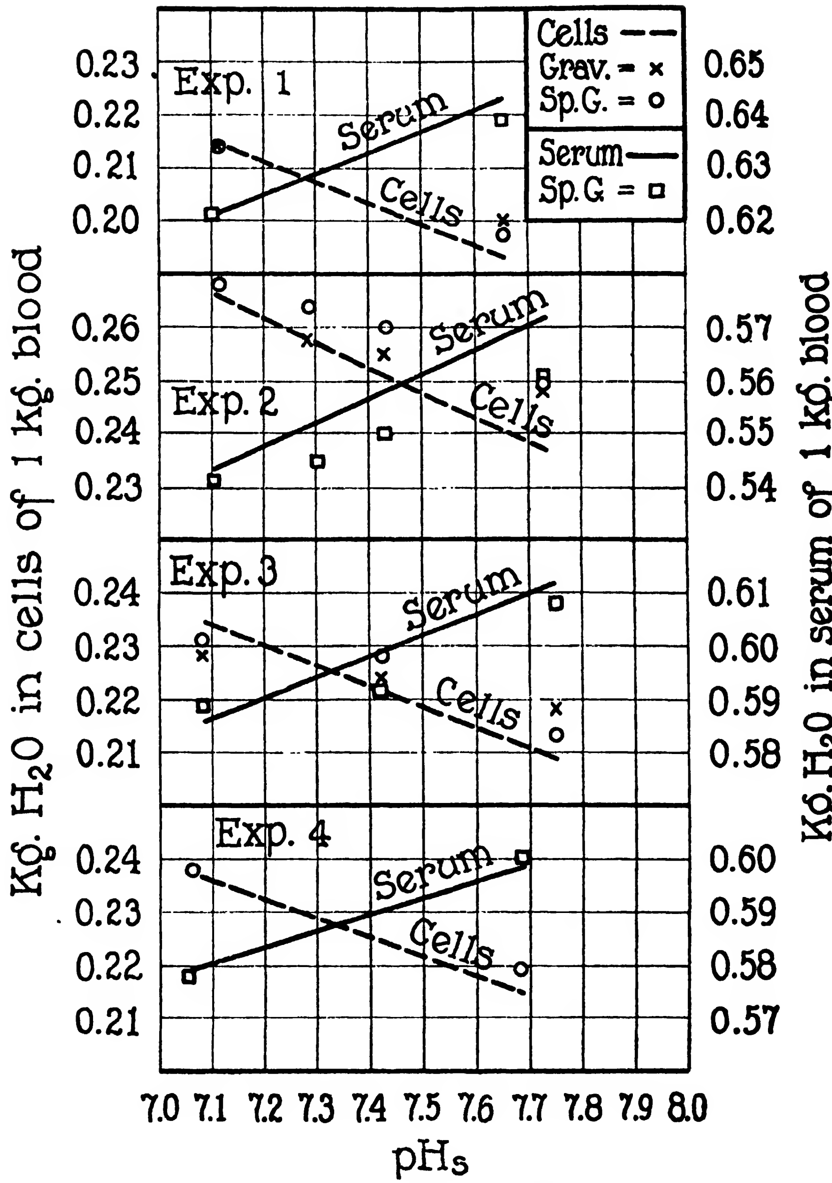

FIG. 13. Comparison of observed and calculated water distribution. Cell and serum water contents calculated by Equations 23 and 24 are indicated by the curves. Water contents observed by the gravimetric and specific gravity methods are indicated by the marked points.

We have continued to use the Sörensen pH unit (0.1 N HCl being assumed to have a pH of 1.090 at 38°) rather than the Bjerrum unit, because practically all biochemical data except Warburg's are at present in terms of the Sörensen unit, and physical chemists do not seem agreed that the Bjerrum unit is preferable.

Equation XX. pH.—pH has been calculated by Hasselbalch's equation as given above (20). The derivation of the value 6.12 for pK′ has been given under the discussion of Equation XIX.

DISCUSSION OF RESULTS.

Since the experimental data have already been referred to in the theoretical part of this paper, little discussion appears necessary at this point.

As stated in our theoretical discussion, since the application of the gas laws to electrolyte solutions is approximate rather than exact, deviations of a few per cent of their values between the Cl and HCO_3 ratios, and of both from the calculated r values, would not be unexpected, nor incompatible with the soundness of the theoretical considerations. The agreement of the two ratios, and of both with the theoretical r values, indicated in Fig. 12, is as close as one might anticipate.

The r values calculated by Equations 10 and 14 respectively (at the bottom of section *d* of Tables VIII, IX, X, and XI) although theoretically equal, differ slightly in most cases, because they are based in part on different analyses, and subject, therefore, to different experimental errors. The value B_c occurs in Equation 14 but not in Equation 10.

The correspondence between the total values of the calculated and observed amounts of water in cells and serum is sufficient to demonstrate the validity of the assumption in Equation 4 on which the theoretical values are calculated, *viz.* that $(H_2O)_s:(H_2O)_c = \Sigma\ (M)_s:\Sigma\ (M)_c$.

The measurement of the water *shift* is, like the chloride ratio, one in which more data, by precise methods, are desirable. The water shifts between cells and serum observed as a result of the changes in CO_2 tension agree, however, except in Experiment 2, with the

theoretically calculated shifts, as indicated in Fig. 13, about as closely as could be expected from the degree of accuracy of the methods and the relatively rather small shifts occurring within the pH range used. Taken together with the agreement between our calculated and Warburg's observed water shifts (observed by noting the change in cell oxygen capacities) reviewed in Table II, the shifts may be considered to be in fair accord with those predicted from the pH changes by Equations 23 and 24.

SUMMARY.

On the basis of the assumption that the laws holding in dilute solutions for (1) the relationships between the reaction and the amounts of base bound by weak and strong acids, (2) the distribution of diffusible and non-diffusible electrolytes on two sides of a membrane (Donnan), and (3) the proportionality between the ratio $\frac{\text{molecules + ions of solute}}{\text{molecules of water}}$ and the osmotic pressure, are also valid for blood, mathematical expressions have been derived which predict the distribution of electrolytes and water between cells and serum, and the manner in which the distribution is affected by changes in pH and oxygen content. The effects of varying CO_2 tensions have been investigated, and the results have been found to approximate those predicted.

Calculations based on the data of Loeb, Atchley, and Palmer (22) indicate the probability that the same physicochemical laws defined for the cells-serum equilibria govern the distribution of electrolytes between blood serum and fluids in the serous cavities, although the permeabilities of the membranes are different and conditions are such that exact osmotic equality cannot apparently exist between the serum and fluids.

Applied to the methods for determining blood pH by the CO_2 capacity and dialysis methods, the data obtained have been used to estimate the corrections necessary because of the heterogeneous character of the blood in the former case, and the Donnan membrane effect in the latter.

In addition to supplying evidence concerning the validity of the theoretical considerations advanced in this paper, the experimental data demonstrate the following:

The base bound by the cell proteins of oxygenated horse blood over the physiological pH range is approximately expressed in milli-equivalents by the equation $[BP]_c = 3.6\ [Hb]_c\ (pH_c - 6.6)$, when Hb expressed millimols of hemoglobin in terms of oxygen capacity. The $[BP]_c$, pH_c curve is slightly concave towards the pH ordinate, so that the above linear equation is an approximate expression of the results.

The base bound by the serum proteins is indicated, over the physiological range of reaction, by the equation $BP_s = 0.068\ P_s\ (pH_s - 4.80)$, where P_s expresses grams of serum protein.

The osmolar concentrations in blood cells and serum are equal when calculated as $\frac{\text{molecules} + \text{ions of solutes}}{\text{water}}$, the electrolytes being assumed to be equally dissociated in cells and serum.

Our thanks are due to Dr. A. B. Hastings and Dr. C. R. Harington for data on the base-binding power of the cell proteins, and to Dr. Hastings for the preparation of the nomogram of Fig. 6*b*.

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THE PRESENCE OF BACTERIA IN THE LUNGS OF MICE FOLLOWING INHALATION.

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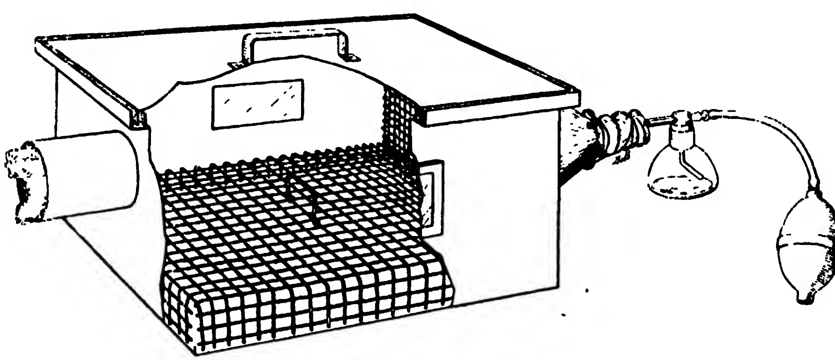
Considerable discussion has arisen at various times as to whether inspired bacteria reach the lungs or are filtered out in the nasal passages. After carefully reviewing the subject of elimination of bacteria from the respiratory tract, Bloomfield (1) concluded that in health and under average conditions of dust exposure microorganisms only occasionally penetrate below the larynx. He thinks that this result is chiefly due to the effective filtration system of the upper respiratory tract. Bacteria inspired into the upper respiratory tract, unless they incite disease, are eliminated by the ciliary currents, die *in situ*, or are carried off through the lymphatics. On the other hand, Jones (2) has recently shown that bacteria may be readily recovered from the borders of lungs removed from healthy calves, rabbits, guinea pigs, white rats, and mice. The microorganisms most frequently observed in cultures from the lungs of these normal animals were streptothrix, molds, and the bacteria of the *subtilis* group. Trillat and Kaneko (3) state that in mice, after 15 minutes of inhalation of a spray containing *Bacillus paratyphosus* and pneumococcus, these organisms are present in the pulmonary alveoli. They found that a much smaller number of paratyphoid bacilli were sufficient to cause infection in mice if they were administered by spraying than if they were mixed with the food or even injected subcutaneously. Wherry and Butterfield (4) have shown that pneumococci may be recovered from the deepest parts of the lungs of mice as long as 18 hours after spraying with a pneumococcus culture. Although they used a virulent Type I pneumococcus culture, none of the twenty-nine mice which were allowed to live, following the inhalation of the pneumococcus mist, became infected.

The purpose of the present study was to extend the above mentioned observations in the hope of obtaining further information concerning pulmonary infection under natural conditions.

Method.

Mice were used as the test animals. They were allowed to inhale an atmosphere in which a fine mist had been produced by spraying a culture of the organism to be studied. The organisms used were pneumococcus, *Streptococcus hæmolyticus*, *Bacillus influenzæ*, and staphylococcus.

The animals were placed in a galvanized iron box 12 by 10 by 7 inches (Text-fig. 1). This box was provided with two glass windows



TEXT-FIG. 1. Spray box.

in the sides and an air vent in one end which was plugged with cotton. At the opposite end was an opening through which the nozzle of an atomizer was inserted. The lid of the box rested in a cotton-filled groove. Before each experiment the entire box was sterilized in an autoclave. After the animals had been placed in the box, 10 to 15 cc. of broth culture were sprayed into the box with a hand atomizer, 10 to 15 minutes continuous spraying being sufficient to fill the interior of the box with a heavy cloud of finely divided droplets. As a rule, the mice were removed from the spray box 1 hour after spraying. At various intervals following the spraying, individual mice were killed and cultures were made from the lungs and other organs. In order to prevent the possibility of aspiration of bacteria from the upper to the

lower respiratory tract at the time of death, the mice were killed instantaneously by clamping the trachea and spinal cord with sponge forceps. The mice were then immersed in a solution of lysol and opened with sterile instruments. As a routine procedure, cultures in broth were made from the heart's blood, the spleen, and the lungs. In making the cultures pieces of the spleen and small pieces of the lung tissue from the borders of the lower lobes were placed directly in tubes of broth. All cultures which subsequently grew were plated on blood agar for further identification. All cultures were incubated

TABLE I.

Recovery of Pneumococci, Hemolytic Streptococci, Influenza Bacilli, and Staphylococci from the Lungs of Mice after Exposure to an Atmosphere Containing Culture in the Form of Spray.

Culture.	No. of mice exposed.	No. of mice killed.	Results of cultures from lungs. Length of time following exposure.										No. of mice not killed.	No. of mice dying with septicemia.	No. of mice surviving.
			1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	8 hrs.	12 hrs.	24 hrs.	48 hrs.			
Pneumococcus	12	6	+	+	−		−		−	−			6	1	5
Streptococcus hæmolyticus	12	6	+	+	+		+		+	+			6	6	0
B. influenzæ	10	6	+	−	+			−			+	−	4		4
Staphylococcus aureus	10	6	+		+	+		+			−	−	4		4

The pneumococcus and streptococcus cultures employed had a virulence such that 0.000001 cc. injected intraperitoneally into mice killed within 36 hours.

The cultures of *B. influenzæ* and *Staphylococcus aureus* had very little virulence on intraperitoneal injection.

for at least 36 hours. In a few instances growth was apparent only after 24 hours incubation. This was probably due to the small number of organisms present.

RESULTS.

In Table I is presented the protocol of one typical experiment. The total number of mice exposed to the atmosphere into which bacteria were sprayed was forty-four. Twelve of the mice were placed in a box into which a culture of pneumococcus was sprayed, twelve in a box into which a culture of *Streptococcus hæmolyticus* was

sprayed, ten in a box into which a culture of *Bacillus influenzæ* was sprayed, and ten in a box into which a culture of *Staphylococcus aureus* was sprayed.

Of the twelve mice exposed to the air containing pneumococci in suspension, six were killed at varying intervals of time, up to 12 hours. In cultures from the lungs of the mice killed 1 and 2 hours after exposure, pneumococci developed, while the cultures from the lungs of the mice killed after 3, 5, 8, and 12 hours remained sterile. Cultures from the spleen and from the heart's blood of all these mice remained sterile. The remaining six mice were not killed but were kept under observation. One died on the 3rd day. No gross lesions of the lungs were present, but cultures showed a general infection with pneumococcus. The other five mice survived and were discarded at the end of 14 days.

Of the twelve mice exposed to the air containing streptococci in suspension, six were killed at the end of 1, 2, 3, 5, 8, and 12 hours, respectively. From the lungs of all these mice streptococci were cultivated, while the cultures from the spleen and heart's blood remained sterile in all cases. Of the remaining six mice which were not killed, all died between the 5th and 10th days and all were found to have a general streptococcus infection.

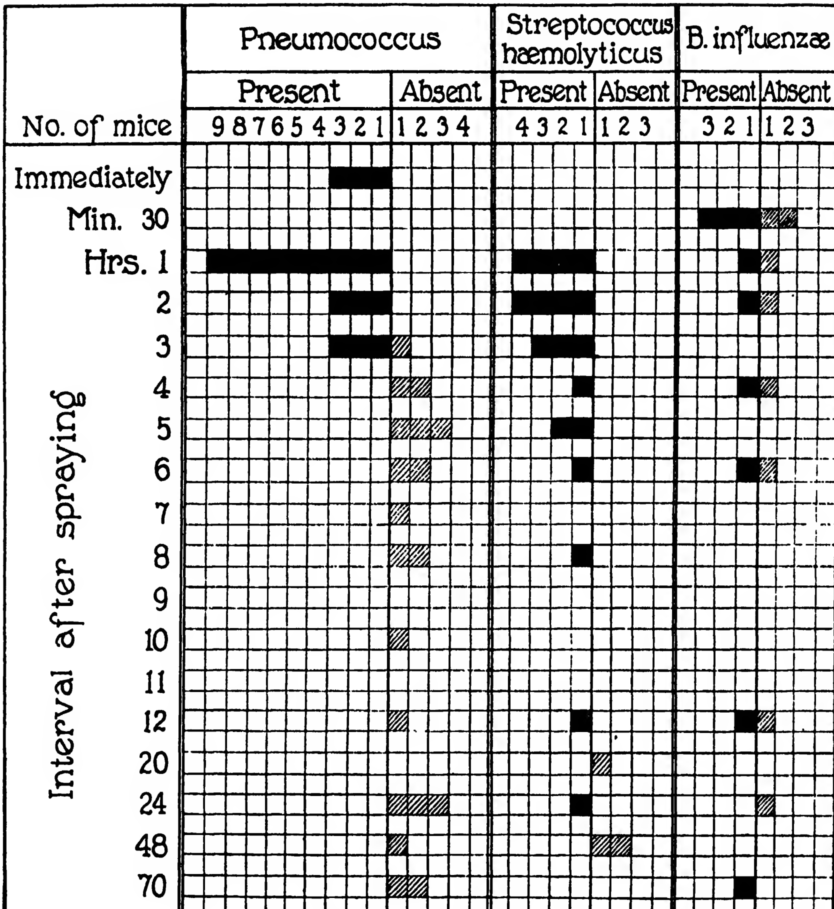
Ten mice were exposed to air containing influenza bacilli in suspension, and six of these were killed at the end of 1, 2, 3, 6, 24, and 48 hours, respectively. The results of the cultures from the lungs in these animals were irregular. From the lungs of the animals killed 1, 3, and 24 hours after spraying, influenza bacilli were cultivated, but the cultures from the lungs of those killed at the end of 2, 6, and 48 hours remained sterile. Cultures from the spleen and heart's blood remained sterile in all instances. The remaining four mice of this group were kept under observation for 10 days and all remained well.

Ten mice were exposed to air containing staphylococci in suspension. Six were killed at varying intervals, and from the lungs of the animals killed 1, 3, 4, and 6 hours after spraying, staphylococci were cultivated, while the cultures from the mice killed 24 and 48 hours after spraying remained sterile. All cultures from the heart's blood and spleen remained sterile. The remaining four mice of this group were not killed but were kept under observation for 10 days. All remained well.

This experiment demonstrates that under the conditions employed, when mice are allowed to breathe an atmosphere in which bacteria have been suspended by spraying, the bacteria reach the lungs and may be found there for considerable periods of time following the spraying. The period during which their presence in the lung may be demonstrated, however, apparently varies with different bacteria. Pneumococci were not found after 3 hours, while streptococci, influenza bacilli, and staphylococci were present after longer intervals. Following inhalation of pneumococcus, only one of the animals which were not killed died later of a general infection, while all the animals exposed to streptococcus spray died later of a general streptococcus septicemia. None of the animals exposed to the *Bacillus influenzæ* or staphylococcus spray suffered from a general fatal infection.

A number of experiments similar to the one described above have been carried out and the results in all instances have in general agreed with those described above. To avoid repetition, the results of all these experiments, including the one just described, have been combined. The total number of the mice exposed to the spray containing virulent Type I pneumococci was 228, those exposed to a spray containing virulent hemolytic streptococci was 45, and those exposed to a spray containing influenza bacilli was 32. No experiments other than that mentioned above have been carried out with staphylococci.

The results of these combined experiments, as far as the animals killed following exposure to the infected spray are concerned, are shown in Text-fig. 2. Of the 228 mice exposed to the spray containing pneumococci, thirty-seven were killed at varying intervals, and the results of the study of these thirty-seven mice are shown on the chart. It is seen that pneumococci were found to be present in the lungs of all of the fifteen mice killed within 2 hours of the exposure. Of four animals killed 3 hours after the exposure, pneumococci were demonstrated in the lungs in three instances, and the cultures from the lungs were negative in one. Of the eighteen mice killed after 3 hours, in the lungs of none could pneumococci be demonstrated. The remaining 191 mice of those exposed, were not killed but were kept under observation for at least 10 to 14 days. Twenty of these mice died, but in sixteen instances the cause of death was not due to pneumococcus infection, as in fourteen the cultures were sterile, and



TEXT-FIG. 2. Recovery of pneumococci, hemolytic streptococci, and influenza bacilli from the lungs of mice following spraying. Each square represents one mouse, the black squares indicating mice in which cultures of the lungs showed bacteria to be present, the cross-hatched squares representing mice in which cultures from the lungs remained sterile.

in two the cause of death was shown to be infection with a Gram-negative bacillus. The remaining four mice, however, died from a general pneumococcus infection, death in these instances occurring on the 3rd to 5th day following the exposure.

Of the forty-five mice exposed to the spray containing streptococci, twenty-one were killed, and the results of the cultures from the lungs in these mice are also shown in Text-fig. 2. It will be seen that of seventeen mice killed before the 12th hour following exposure, streptococci were demonstrated to be present in the lungs of all, and in one mouse killed after 24 hours streptococci were also cultivated from the lungs. In the three other mice, one killed after 20 hours and two after 48 hours, the cultures from the lungs remained sterile. The remaining twenty-four mice of the streptococcus series were not killed but were kept under observation. In striking contrast to the mice exposed to pneumococcus spray, seventeen, or 70 per cent, of these mice died between the 2nd and 12th days, and all suffered from a general streptococcus infection. Only seven mice were living at the end of 14 days.

Of the total thirty-two mice exposed to the spray containing influenza bacilli, seventeen were killed. Again the results of lung culture are shown in Text-fig. 2. It is obvious that here the results are irregular, in some instances the cultures from the lungs being positive and in others negative, both in animals killed soon after exposure and in others killed after much longer periods. The fifteen mice which were not killed were kept under observation for 14 days and none died during this period.

DISCUSSION.

The results obtained by combining all the experiments support the correctness of the previous observations. That during exposure of mice to a dense spray containing bacteria in suspension, the bacteria reach the lungs, seems to be demonstrated. The exact degree of penetration is as yet uncertain, but as the bacteria can be grown from very minute portions of lung tissue removed from the periphery, it seems safe to conclude that the bacteria reach the finer bronchi at least.

The further history of the bacteria which have penetrated the lungs seems to depend upon the variety of microorganism concerned. As regards pneumococcus, the bacteria are disposed of within a few hours. On the question as to whether they are killed *in situ*, are taken up by leucocytes, or are carried to other parts of the body, the present experiments throw no light. Streptococci, on the other hand,

at least with the cultures employed in these experiments, persist in the lungs for a much longer period of time. This fact may be associated with the occurrence of true infection, as subsequent events in many of these animals indicate. The results of the experiments in which *Bacillus influenzæ* was employed were more irregular; apparently sometimes these bacteria remain in the lungs for considerable periods of time, in other instances they disappear very quickly. Whether this result is associated with failure of the bacteria to penetrate deeply, or is related to the difficulty of cultivation of this organism, is not certain.

The fact that the penetration of the lungs by streptococci is usually followed by a general infection, while pneumococci are disposed of without any general infection resulting has been somewhat surprising. This was especially so in view of the fact that the virulence of the pneumococcus culture employed, as tested by intraperitoneal injection into mice, was equal to that of the streptococcus culture.

It was suggested that the apparent lack of invasiveness of the inhaled pneumococci might depend upon an insufficient number of organisms reaching the lung. That this view, however, is not tenable is shown by the following experiment. Six mice were exposed to an atmosphere containing pneumococci in suspension as previously described, and after 1 hour had elapsed all were killed. A single lobe of one lung was removed from each of the mice and each lobe was ground up separately in a mortar with sterile sand and normal salt solution. The supernatant fluid was then used for inoculation intraperitoneally into six normal mice. Five of the six mice injected died within 48 hours with a general pneumococcus infection. One mouse survived. It is evident, therefore, that in most instances pneumococci reach the lung in sufficient numbers to cause infection provided other conditions are favorable. It seems probable that normally the conditions in the lungs of mice are unfavorable for infection with pneumococci, even when the number of organisms reaching the lower respiratory tract is considerable. It is true that Blake and Cecil (5) infected monkeys with pneumococci by injecting minute amounts of the culture into the trachea, but even in these experiments a relatively considerable amount of fluid (1 cc.) was injected. The relation of the

injury produced by the penetration of the needle through the skin and tracheal mucous membrane to the subsequent infection should also be borne in mind.

In any case the present experiments indicate that in mice at least the mere presence of pneumococci in the lungs is not sufficient to induce infection, but that other factors are also concerned. An attempt has been made to discover something of the nature of these other factors by exposing the mice to altered external conditions before exposing them to the atmosphere of the spray box. The effects of sudden chilling and exposure to cold were studied. Mice were chilled before being placed in the spray box, others were kept chilled while they were in the box, and still others were chilled after the exposure in the box had taken place. They were chilled by being dipped into ice water, placed in an ice box, sprayed with cold water, or allowed to stand in melting snow. None of these procedures resulted in so changing conditions that infection occurred with any greater frequency than in the controls.

Attempts to lower local resistance by inducing slight injuries to the mucous membrane were also made. These attempts consisted in allowing animals to inhale ether before or after exposure to the spray, and certain mice were also exposed to an atmosphere containing finely divided quartz sand or talc in the form of dust before or following the exposure. These experiments also resulted in failure to increase the frequency of infection.

The present experiments, therefore, fail to shed any light on the exact mode of natural infection of the lung with pneumococci. They indicate that even in so susceptible an animal as the mouse other factors than the presence of the pneumococci in the lung are necessary for infection.

CONCLUSIONS.

1. When mice are exposed to an atmosphere containing cultures of bacteria in the form of a fine mist, the bacteria readily penetrate into the lower respiratory tract.

2. Pneumococci which have reached the lung as a result of this procedure usually disappear within a few hours and give rise to no

infection. Hemolytic streptococci, on the other hand, persist in the lung for a considerably longer time and a general septicemia usually follows.

3. Attempts to determine the conditions under which pneumococci which have reached the lung by inhalation may induce a local or general infection have not been successful.

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STUDIES ON THE ENZYMES OF PNEUMOCOCCUS.

IV. BACTERIOLYTIC ENZYME.

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In preceding papers (1), studies on the proteolytic, lypolytic, and carbohydrate-splitting enzymes of pneumococcus have been reported. It has been shown that these enzymes are intracellular in nature and can be extracted from the bacterial cell by various methods. They possess to a remarkable degree the power of actively hydrolyzing peptones to simpler peptides and amino-acids, of converting carbohydrates to simpler products, and of splitting esters to fatty acids.

In addition to the enzymes previously described, it has been found that solutions of the cellular substance of pneumococci also contain an enzyme which is capable of exerting a lytic action upon other dead bacterial cells.

Methods.

Preparation of Enzyme.—In previous papers, the bacteriological and chemical methods used in these studies have been described in detail (1). The same methods have been followed in the present investigation. In the preparation of active enzyme-containing solutions from pneumococci the bacterial cells have been dissolved in bile, or the intracellular substances have been extracted by autolysis. When the former method is used the presence of bile does not obscure the effects of the bacteriolytic enzyme upon heat-killed organisms inasmuch as bile itself does not cause lysis of pneumococci which have been killed by heat.

Preparation of Substrate.—The substrates have consisted of suspensions of washed bacteria in phosphate solution of known hydrogen ion concentration. The bacterial suspensions were exposed to a temperature of 60°C. for 30 minutes (water bath), or 120°C. for 20 minutes

(autoclave). Antiseptics were not used as preservatives, and the sterility of all enzyme-substrate mixtures was proved by subculture.

Action of Intracellular Enzymes of Pneumococcus on Heat-Killed Bacteria.

Experiment 1.—Enzyme: The bacterial residue from 2 liters of a 10 hour plain broth culture of Pneumoccocus Type II was washed with physiological salt solution and resuspended in 10 cc. of 0.1 M phosphate mixture of pH 6.2. The bacterial suspension was immediately frozen and thawed seven times, and kept at ice box temperature for 10 days. During this period cytolysis occurred and the bacterial detritis settled to the bottom, leaving a clear, slightly opalescent supernatant fluid which was used in this experiment. 0.1 cc. of this solution plated on blood agar showed no growth after several days incubation.

Substrate: Bacterial substrates were prepared by suspending the washed cells in 0.1 M phosphate solutions of pH 7.8. The following organisms were tested: Pneumococci Types I and II, *Streptococcus viridans*, *Streptococcus hæmolyticus*, and *Staphylococcus aureus*. The bacterial suspensions were of about equal opacity and were autoclaved 20 minutes at 15 pounds pressure before use. 0.1 cc. of the active enzyme solution was added in each instance to 0.5 cc. of the bacterial substrate, and the total volume made up to 1 cc. by the addition of 0.1 M phosphate solution of pH 7.8. The tubes were then placed in a water bath at 37°C., and the degree of bacteriolysis was noted at varying intervals by observing the relative opacity of the tubes, and by microscopic examination of stained films.

After 15 minutes incubation at 37°C., tubes containing a mixture of active enzyme and heat-killed pneumococci showed marked disintegration of the bacterial bodies. After 2 hours incubation, complete dissolution occurred and stained films showed only Gram-negative detritis, and shadow forms of the organisms. Films prepared from the tubes containing pneumococcus enzyme and the dead bodies of *Streptococcus viridans* showed at this period many Gram-negative organisms in unevenly stained chains, with disintegration of over half of the bacterial cells. On the other hand, stained films of the tubes in which the active enzyme was in contact with substrates of hemolytic streptococci or staphylococci showed no visible change in the organisms either in form, size, or staining properties. The pneumococci used as substrate in this experiment were of another type than those from which the enzyme was derived. This fact would indicate that the bacteriolytic enzyme is not type-specific, since it exerts a lytic action on strains of pneumococci serologically unrelated. The bacteriolytic effect of pneumococcus enzyme on *Streptococcus viridans*, although distinct, is much slower, being complete only after the action is continued for several hours. On the other hand, the strains of *Streptococcus hæmolyticus* and *Staphylococcus aureus* studied in the present investigation were uninfluenced by the action of the pneumococcus enzyme, and even after prolonged exposure showed no visible changes morphologically or tinctorially.

In controls, in which no enzyme had been added to the suspensions of heated bacteria, the organisms were still of normal appearance and gave no evidence of changes in coloring or form even after incubation for 20 hours at 37°C. Pneumococci acted upon by enzyme for this length of time appeared only as Gram-negative amorphous material; the green-producing streptococci were at this stage undergoing further disintegration, in stained films only an occasional Gram-positive form was seen, and the majority of the organisms appeared as faint Gram-negative shadows. Even after prolonged action of the pneumococcus enzyme on the substrate, the hemolytic streptococci and staphylococci were well preserved, retained their staining properties, and showed no evidence of bacteriolysis. After incubation for 48 hours, the tubes in which active bacteriolysis had occurred, namely those containing pneumococci, and streptococci of the *viridans* type, showed evidence of clearing with disappearance of the bacterial whirl, and were much more translucent than the control tubes to which no enzyme had been added. In the case of hemolytic streptococci and staphylococci no noticeable change in the gross appearance of the digestion mixture was evident.

From this experiment it appears that pneumococci possess an active intracellular agent which has the property of causing lysis of the bodies of heat-killed pneumococci, and, furthermore, that the lytic agent is not type-specific, since strains of pneumococci serologically different are equally affected. The bacteriolytic enzyme of pneumococcus also possesses to considerable degree the ability to attack streptococci of the *viridans* variety, cocci which biologically are more closely related to pneumococci than are hemolytic streptococci and staphylococci. Cocci of the last two varieties are apparently unaffected by the action of the enzyme, even after prolonged contact.

Influence of Hydrogen Ion Concentration on the Activity of the Intracellular Bacteriolytic Enzyme of Pneumococcus.

Under conditions similar to those described in Experiment 1, the effect of varying the hydrogen ion concentration on the bacteriolytic action of the enzyme was tested. In this experiment the enzyme solution was prepared from pneumococci of Type I and the bacterial substrate from pneumococci of Type II. The washed bacterial cells from 150 cc. of a plain broth culture of Pneumococcus Type II were suspended in 5 cc. of 0.1 M phosphate solution pH 7.4, and autoclaved at 15 pounds pressure for 20 minutes. After being killed by heat, the organisms were again centrifuged and the bacterial residue was washed and resuspended in 2 cc. of sterile distilled water. 0.1 cc. of active

enzyme solution and 0.1 cc. of bacterial substrate were now added to tubes each containing 1 cc. of 0.1 M phosphate solution. The tubes were adjusted to the desired hydrogen ion concentration by the addition, in the more acid ranges, of hydrochloric acid. The results of this experiment are shown in Table I.

TABLE I.

Influence of Hydrogen Ion Concentration on the Activity of the Bacteriolytic Enzyme of Pneumococcus.

pH	Enzyme solution of Pneumococcus Type I.	Substrate of heat-killed Pneumococcus Type II.	0.1 M PO_4 solution.	Lysis after 2 hrs. at 37°C.
	cc.	*cc.*	*cc.*	
2	0.1	0.1	0.8	−
	0	0.1	0.8	−
3	0.1	0.1	0.8	−
	0	0.1	0.8	−
4	0.1	0.1	0.8	−
	0	0.1	0.8	−
5	0.1	0.1	0.8	++
	0	0.1	0.8	−
6	0.1	0.1	0.8	+++
	0	0.1	0.8	−
7	0.1	0.1	0.8	+++
	0	0.1	0.8	−
7.8	0.1	0.1	0.8	+++
	0	0.1	0.8	−
8	0.1	0.1	0.8	+++
	0	0.1	0.8	−

+++ indicates complete lysis; ++, marked lysis; −, no lysis.

The lytic action of the enzyme was evident in the zone pH 5 to 8. At reactions more acid than pH 4 there was no evidence of lysis. At pH 3 a marked precipitation occurred. This may possibly be attributed to acid agglutination which for Type II pneumococci occurs at about this reaction. The optimum zone of hydrogen ion concentra-

tion, for the action of the bacteriolytic enzyme, corresponds closely to that already described for the peptonase, lipase, invertase, and inulase of pneumococcus.

Influence of Concentration of Enzyme on the Activity of the Intracellular Bacteriolytic Enzyme of Pneumococcus.

That the lytic action of solutions containing the intracellular substances of pneumococcus is enzymotic in nature, is evidenced by the following experiment in which it is shown that the rate and amount of lysis are a function of the concentration of the enzyme present. The conditions of this experiment were similar to those already described in previous experiments; the enzyme-containing solution was prepared

TABLE II.

Influence of Concentration of Enzyme on the Activity of the Bacteriolytic Enzyme of Pneumococcus.

Enzyme solution of Pneumococcus Type II.	Substrate of heat-killed Pneumococcus Type II.	0.1 M PO_4 solution pH 7.8	Lysis after 1 hr. at 37°C.
cc.	*cc.*	*cc.*	
0.1	0.5	0.5	++++
0.05	0.5	0.5	+++
0.025	0.5	0.5	++
0.0125	0.5	0.5	+
0.006	0.5	0.5	+
0.003	0.5	0.5	+
0	0.5	0.5	0

++++ indicates complete lysis; 0, no lysis.

from Pneumococcus Type II. This particular enzyme preparation had been kept in the ice box for 27 days. The bacterial substrate consisted of Type II pneumococci which had been prepared as already described.

From Table II it is evident that the action of the intracellular bacteriase is directly proportional to the concentration of the enzyme. It is interesting to observe that the activity of this enzyme, even after preservation in cold for 4 weeks, was still pronounced in quantities as small as 0.025 cc., and that traces of its action were detected in minimum amounts of 0.003 cc.

Influence of Heat on the Activity of the Intracellular Bacteriolytic Enzyme of Pneumococcus.

The following experiment was carried out to determine the effect of heat upon the bacteriolytic enzyme of pneumococcus.

300 cc. of plain broth pH 7.8 were seeded with a large inoculum (0.5 cc.) of a culture of Pneumococcus Type II. After 8 hours growth at 37°C., the culture was centrifuged and the bacteria were suspended in 20 cc. of 0.1 M phosphate solution at pH 7.2. To 10 cc. of this bacterial suspension, representing the cells from 150 cc. of culture, 0.2 cc. of undiluted ox bile was added. Solution of the organisms was marked after 30 minutes in the water bath at 37°C. and was complete after standing in the ice box over night. The remaining 10 cc. of the bacterial suspension to be used as substrate were autoclaved at 15 pounds pressure for 10 minutes in order to kill the organisms and to destroy the intracellular ferments.

TABLE III.

Influence of Heat on the Activity of the Bacteriolytic Enzyme of Pneumococcus.

Enzyme solution of Pneumococcus Type II, 0.5 cc.	Substrate of heat-killed Pneumococcus Type II.	Lysis.	
		After 2 hrs. at 37° C.	After 18 hrs. at 37° C.
	cc.		
Heated at 60°C. for 30 min.	0.5	–*	–
" " 80° " " 30 "	0.5	–	–
" " 100° " " 30 "	0.5	–	–
Unheated	0.5	++	++
No enzyme; phosphate solution and bile (1:50)†	0.5	–	–

* – indicates no lysis; ++, complete lysis.

† This shows that bile alone has no lytic effect on heat-killed pneumococci.

Aliquot portions of the solution containing the dissolved bacteria were heated in a water bath for 30 minutes at 60°, 80°, and 100°C., respectively. These heated solutions in 0.5 cc. amounts were now added to equal quantities of the suspension of heat-killed pneumococci. The results of this experiment are shown in Table III.

Analysis of Table III shows that exposure in the water bath to a temperature of 60°C. for 30 minutes destroys the activity of the bacteriolytic enzyme of pneumococcus. The resistance of the bacteriolytic enzyme of pneumococcus to heat is markedly less than that of the lytic agent of *Bacillus pyocyaneus* which, according to Emmerich (2), withstands steam at 100°C. for 2 hours.

DISCUSSION.

Evidence has been presented in this paper that pneumococci contain a bacteriolytic enzyme. This enzyme is associated with a number of other active intracellular agents which exert their effect upon various substances and which because of their enzymotic nature have been described as the intracellular peptonase, lipase, inulase, and invertase of pneumococcus. The bacteriolytic enzyme possesses the property of causing lysis of the dead bacterial bodies of pneumococci, and to a less extent the disintegration of closely allied organisms, such as *Streptococcus viridans*. However, it has no effect upon certain other Gram-positive cocci, as *Staphylococcus aureus* and *Streptococcus hæmolyticus*. The enzyme is not type-specific in its action, since an enzyme solution prepared from pneumococci of one type exerts a comparable action upon pneumococci of a heterologous type.

Emmerich, Löw, and Korschun (3) have demonstrated an enzyme in cultures of *Bacillus pyocyaneus* which possesses a remarkable lytic power. This enzyme, pyocyanase, in extraordinarily small amount, is capable of causing lysis of a number of other microorganisms such as *Bacillus diphtheriæ*, *Vibrio choleræ*, *Bacillus typhosus*, *Bacillus pestis*, streptococcus, and staphylococcus. In contrast with the pneumococcus enzyme, the enzyme from *Bacillus pyocyaneus* manifests its action on the living bacterial cell. Pyocyanase is remarkably heat-stable, resisting a temperature of 100°C. for 2 hours (2). This lytic agent is considered by Emmerich and his coworkers to be different from the peptonizing enzyme of *Bacillus pyocyaneus*. Löw and Kozai (4) have also demonstrated a bacteriolytic enzyme in cultures of *Bacillus prodigiosus*. Emmerich and Löw (5) include these bacteriolytic enzymes in the group of nucleases, which act on the nucleoprotein of the bacterial cell.

In the present instance, no assumption is made as to the identity of the pneumococcus bacteriolytic enzyme, since it is not known what particular constituent or constituents of the bacterial cell are acted upon. Whether lysis of pneumococci under these circumstances is the result of a single enzyme or the product of the interaction of more than one, and whether the enzyme or group of enzymes concerned in autolysis of pneumococci play any part in this form of lysis are questions at present undecided.

The fact that the pneumococcus bacteriolytic enzyme apparently has no effect on the living cell suggests that the living organism may possess a protective mechanism, possibly of the nature of antiferment. It may be only when this mechanism has been interfered with or destroyed that the bacterial cell is exposed to the action of its own enzymes.

SUMMARY.

1. Pneumococci possess an active intracellular enzyme which causes lysis of heat-killed pneumococci of the same and heterologous types and to a less degree of a closely related organism, *Streptococcus viridans*.
2. The optimum reaction for lysis lies between pH 6 and 8.
3. The bacteriolytic action is proportional to the concentration of the enzyme.
4. Heating the enzyme for 30 minutes at 60°C. destroys its activity.
5. The possible relation of the enzyme to autolysis is discussed.

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STUDIES ON BACTERIAL NUTRITION.

IV. EFFECT OF PLANT TISSUE UPON GROWTH OF PNEUMOCOCCUS AND STREPTOCOCCUS.

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(Received for publication, April 12, 1923.)

Study of the growth requirements of *Bacillus influenzæ* (1–4) indicated that for the cultivation of this organism at least two substances must be present in the culture medium. Both of these substances are present in red blood corpuscles. One, a vitamine-like substance, can be extracted not only from red blood corpuscles but from yeast and vegetable cells as well, and is relatively thermolabile. This substance has been called the V factor. The other substance is also present in red blood corpuscles and in plant tissue, but it is thermostable and is effective in amounts so small as to suggest the possibility that it functions as a catalyst. It has been called the X factor. These observations on the growth of *Bacillus influenzæ* in the absence of blood have been extended (5). The organism can be readily grown in plain broth in which small pieces of sterile, unheated yellow or white turnip, carrot, beet, parnsip, or sweet potato have been placed. Knowledge of the growth-stimulating property of unheated plant tissue for organisms as sensitive to cultural conditions as the bacteria of the hemophilic group encouraged an extension of the study to other groups of bacteria. The present paper presents the results of a study of the effect of plant tissue upon the growth of certain Gram-positive cocci.

Material.

Media.—The plant tissues used in this study were white potato, sweet potato, carrot, white turnip, yellow turnip, parsnip, beet, and banana. Care was observed in selecting fresh young specimens. With the same technique described in a previous communication (4),

sterile pieces of these vegetables were placed in phosphate broth of pH 7.8. When air is not excluded from media prepared in this way oxidation of the plant tissue results in brown discoloration of the vegetable and of the supernatant broth. To prevent this the media were sealed with sterile vaseline. The media could then be stored in the ice box for many weeks without discoloration.

Organisms.—Three strains of pneumococci, three of hemolytic streptococci, and five of non-hemolytic streptococci were employed in these experiments.

Growth of Pneumococcus in Media Containing Sterile Plant Tissue.

The rate of growth of pneumococcus in broth is dependent upon several factors, the most important of which are (*a*) the growth activity of the bacteria used for seeding (6), (*b*) the size of the inocula, and (*c*) factors pertaining to the media such as its pH (7), its content in nutritive substances, etc. Depending upon the balance between these factors, growth as determined by the number of viable organisms present at different intervals during the life of the culture is divisible into four periods: (*a*) period of lag, during which the number of viable organisms in the culture decreases, remains constant, or slowly increases; (*b*) logarithmic period, in which the culture maintains its maximum rate of growth; (*c*) stationary period, in which the maximum rate of growth is not maintained, the organisms present, though viable, reproducing more slowly or not at all; and (*d*) period of decline, in which the number of viable organisms decreases rapidly, the pneumococcus culture eventually becoming sterile (6).

Effect of the Addition of Sterile Unheated Potato on the Growth of Pneumococcus.

With the above facts in mind, an experiment was planned to show what effect the addition of sterile unheated plant tissue to fluid media would have on the growth of pneumococci. The period of lag was first studied. Plain broth containing sterile unheated potato was used as the test medium. As a control, broth to which 1 per cent of dextrose had been added, was employed. Dextrose is readily fermented by pneumococcus, and is known to accelerate the growth of this organism.

Experiment 1.—Into a 300 cc. flask containing 150 cc. of phosphate meat infusion broth (pH 7.6) were placed four cylindrical pieces of unheated, sterile potato, each about 1.5 cm. long and 0.5 cm. in diameter. The flask was then incubated at 37°C. to insure sterility. A control flask, containing 150 cc. of broth of the same lot, to which 1 per cent dextrose had been added, was prepared. With the media at 37°C. each flask was seeded with 0.1 cc. of a 6 hour broth culture of Pneumococcus Type I. The culture used for seeding was actively growing, as indicated by the presence of long chains in the stained film. At frequent intervals over a period of 9 hours, fractions of both the dextrose broth and the potato broth cultures were removed and estimations made of the number of viable organisms present in each. In making these estimates the following method was employed. Several accurate dilutions of the culture were made. 0.5 cc. from each of two or more of these dilutions was added to a tube of 1 per cent dextrose agar and plates were poured. The necessary calculations for the different dilutions having been made, the average

TABLE I.

Acceleration of Growth of Pneumococcus in Broth Containing Sterile Unheated Potato.

Time after seeding.	Log of No. of colonies per 0.5 cc. of culture.	
	Plain broth + potato.	Plain broth + 1 per cent dextrose.
hrs.		
0	3.7	3.8
1	4.0	4.1
2	4.2	4.0
3	4.9	3.8
4	5.1	
5	5.8	4.0
6	6.3	4.0*
7	7.1	4.0*
9	7.9	4.0*

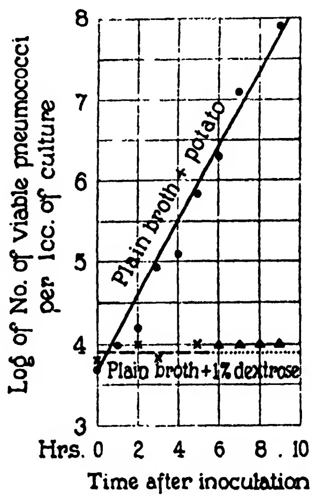
* No colonies in the dilutions plated. Therefore the exact determination was not made. The log is less than 4.

of the number of colonies present in the plates after 48 hours at 37°C. was used for the colony count per 0.5 cc. of culture for that hour. The results of these estimations as expressed in the logarithms of the number of colonies present at the time of each examination, are given in Table I. By using these logarithms as ordinates and the hours of examination as abscissæ, Text-fig. 1 was constructed.

From Table I and Text-fig. 1 it is evident that the organisms in broth containing sterile unheated potato grew steadily and at a constant rate. There occurred no period of lag. In contrast with this,

organisms seeded into broth containing an abundance of an easily fermentable substance, dextrose, but no plant tissue, failed to attain this maximum rate of growth for a period of at least 9 hours.

A second experiment was now performed in order to extend these observations and to determine the effect of the presence of unheated plant tissue upon the rate of growth during the entire cycle of a pneumococcus broth culture and also the effect upon changes in reaction which usually occur in the medium during growth. It is



TEXT-FIG. 1. Influence of sterile unheated plant tissue on the acceleration of growth of pneumococcus. ▲ indicates less than 4.

known that during growth of pneumococci in broth an increase in the pH occurs. When the medium is enriched by the addition of readily fermentable substances, as dextrose or certain other sugars, the acid production by the growing pneumococcus is still more marked (8).

Experiment 2.—The medium was prepared as in Experiment 1. Two 300 cc. flasks, one containing 150 cc. of 1 per cent dextrose broth, the other 150 cc. of plain broth to which had been added about 5 gm. of sterile unheated potato, were seeded with small identical amounts of a growing broth culture of Pneumococcus Type I. At intervals, as noted in Table II, samples were removed from each of the flasks. Estimations of the number of viable organisms were made, as in Experiment 1. Determinations of pH, by means of the colorimetric method, were made at the same time. The results of these observations are shown in Table II, and are graphically represented in Text-fig. 2.

From Table II and Text-fig. 2 it is seen that, under the conditions of the experiment, no period of lag occurred in the broth culture containing unheated potato. The control culture in dextrose broth, a medium quite favorable for prompt and abundant growth of pneumococcus when the inoculum is sufficiently large, showed a period of lag of 18 hours, during which the viable organisms in the culture apparently decreased in number. However, when once the culture

TABLE II.

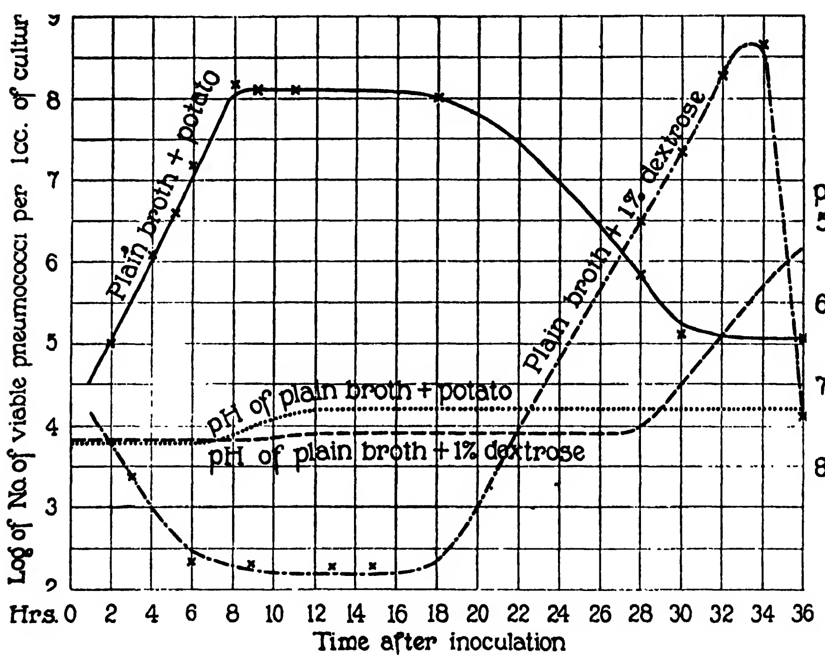
Comparison of Growth of Pneumococcus in Potato Broth and Dextrose Broth.

Time after seeding.	Broth containing unheated potato.		Broth containing 1 per cent dextrose.	
	Log of No. of colonies per 0.5 cc. of culture.	pH of culture.	Log of No. of colonies per 0.5 cc. of culture.	pH of culture.
hrs.				
1	4.5	7.7	4.2	7.7
2	5.0			
3	5.5		3.3	
4	6.1			
5	6.7	7.7		7.7
6	7.3		2.3	
8	8.2	7.5		7.65
9	8.1		2.3	
11	8.1			
13	8.1	7.3	2+*	7.6
15	8.1		2+*	
18	8.0		2.3	
28	5.8	7.3	6.5	7.6
30	5.1	7.3	7.4	7.1
32			8.4	6.3
34			8.7	5.3
36	5.1	7.3	4.1	

* Actual number of colonies not determined.

entered upon the logarithmic phase of growth, the generation time was approximately the same in the dextrose broth culture as in the potato broth culture; that is to say, cell division, when once initiated, proceeded at about the same rate in the two cultures. It is noteworthy that the stationary phase of the pneumococcus culture in potato broth extended over a period of 10 hours, while that of the culture in dextrose broth was of extremely short duration.

Other observations have shown that in the potato broth culture there is a prolongation of the period of decline in the growth curve; that is, the period in which cell death is proceeding at a more rapid rate than multiplication. It is especially prolonged when compared with a culture in dextrose broth. Moreover, when young actively growing potato broth cultures are removed from the incubator and are placed on ice, the bacteria may remain viable for very long periods



TEXT-FIG. 2. Influence of sterile unheated plant tissue on the growth curve of pneumococcus.

of time, even 8 months. These facts indicate that not only is the initiation of growth accelerated in the potato broth, but death of the bacteria occurs more slowly in this medium.

Effect of the Addition of Other Sterile Unheated Plant Tissues on the Growth of Pneumococcus.

The following experiment illustrates the effect on growth of pneumococcus when vegetable tissue, other than potato, is added to plain broth.

Experiment 3.—Sterile pieces of parsnip, sweet potato, carrot, yellow turnip, white turnip, and banana, each weighing about 0.5 gm., were placed in tubes containing 10 cc. of plain broth and these were sealed with vaseline. After incubating several days to insure sterility, the seals were removed and 2 cc. of the broth were taken from each tube for colorimetric pH determinations. Each tube was then seeded with 0.05 cc. of an actively growing broth culture of Pneumoccocus Type II. A tube containing 8 cc. of broth of the same lot, but no vegetable tissue, was seeded with the same amount of the same culture. The tubes were placed in the incubator at 37°C. Observations on the degree of clouding present in the media were made after 5 hours, and again after 17 hours incubation. The results are given in Table III.

TABLE III.

Acceleration of Growth of Pneumococcus in Broth Containing Sterile Unheated Vegetable Tissue.

Media.			Inoculum.	Growth of pneumococcus.*	
Broth.	Plant tissue.	pH at time of seeding.		5 hrs.	17 hrs.
cc.			*cc.*		
8	None.	7.8	0.05	–	++
8	Parsnip.	6.3	0.05	+	++++
8	Banana.	6.4	0.05	+++	++++
8	Sweet potato.	6.7	0.05	++	++++
8	Carrot.	6.9	0.05	++	++++
8	Yellow turnip.	7.1	0.05	++	++++
8	White "	7.2	0.05	±	++++
8	Potato.	7.4	0.05	+++	++++

* – indicates no macroscopic growth; ±, faint haze confined to vicinity of plant tissue; +, faint clouding throughout media; ++, moderate clouding throughout media; +++, marked clouding throughout media; ++++, maximum growth attained.

From Table III it is evident that other plant tissues have the same growth-stimulating effect upon pneumococcus that potato possesses. It is also evident that this stimulation operates in spite of the fact that the initial reaction of the media may be unfavorable for growth of this organism.

The Limiting pH for the Growth of Pneumococcus in Potato Broth.—It has been shown that the optimum hydrogen ion concentration for growth of pneumococcus is pH 7.8 and that growth in plain broth

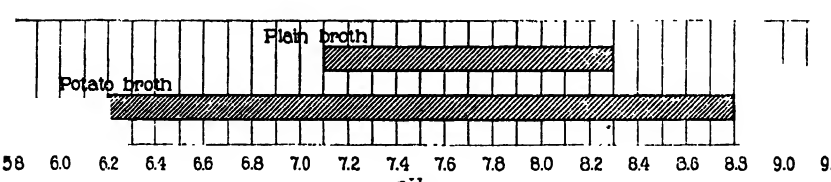
can be initiated only if the hydrogen ion concentration is between pH 7 and 8.3 (7). The observations made in Experiment 3 indicate that if there is present in the medium a bit of unheated vegetable tissue growth of pneumococcus can be initiated even though the hydrogen ion concentration is only pH 6.3. An attempt was made, therefore, to determine the limiting hydrogen ion concentrations for the initiation of growth of pneumococcus in broth containing sterile unheated potato.

TABLE IV.

Growth of Pneumococcus at Different Hydrogen Ion Concentrations in Broth Containing Sterile Unheated Plant Tissue.

Inoculum.	pH of media.	Growth of pneumococcus in plain broth after 18 hrs.*	Growth of pneumococcus in plain broth + potato after 18 hrs.*
cc.			
0.05	5.8	–	–
0.05	6.2	–	+
0.05	6.8	–	+
0.05	7.1	+	+
0.05	8.3	+	+
0.05	8.6	–	+
0.05	8.8	–	+
0.05	9.0	–	–

* – indicates no macroscopic growth; +, growth.



TEXT-FIG. 3. Influence of sterile unheated plant tissue on the range of hydrogen ion concentration for initiation of growth of pneumococcus.

Experiment 4.—Tubes containing 10 cc. of potato broth were prepared in the usual way and incubated to insure sterility. In a number of tubes the hydrogen ion concentration was adjusted, by the addition of suitable amounts of sterile N and 0.1 N HCl or NaOH, so that a series of tubes was obtained in which the reaction ranged between pH 5.8 and 9. In the same way there was prepared a series of tubes of plain broth, without potato, in which the hydrogen ion concentration ranged between the same points. All these tubes were then seeded, each with

0.05 cc. of a growing culture of Pneumococcus Type I, and incubated at 37°C.[1] Growth of pneumococcus occurred as is recorded in Table IV, and as represented diagrammatically in Text-fig. 3.

The results in Table IV indicate that the presence of sterile unheated potato in broth greatly increases the range of pH in which the growth of pneumococcus can be initiated. This increased range extends between pH 6.2 and 8.8.

Effect of the Addition of Sterile Unheated Potato on the Growth of Hemolytic and Non-Hemolytic Streptococci.

In order to determine whether unheated plant tissue might exert a growth-stimulating effect on other cocci, strains of hemolytic and of non-hemolytic streptococci were grown in plain broth alone and in the same broth to which unheated plant tissue had been added. Differences in growth activity of the same organisms in the two types of media were noted.

TABLE V.

Acceleration of Growth of Hemolytic and Non-Hemolytic Streptococci in Broth Containing Sterile Unheated Plant Tissue.

Organisms.	Growth in 10 cc. of plain broth.*	Growth in 10 cc. of potato broth.*
Results after 14 hrs. incubation.		
1. Hemolytic Streptococcus 3/22	−	+++
2. " " 23/20	−	+++
3. " " 84/18	−	+++
Results after 18 hrs. incubation.		
4. Non-hemolytic Streptococcus A141	−	++
5. " " A135	−	+
6. " " B39	+	+++
7. " " 38d	±	+++
8. " " A149	±	++

* − indicates no macroscopic growth; ±, faint haze; +, bacterial whirl; ++, marked growth; +++, maximum growth.

[1] A set of uninoculated tubes containing potato broth at reactions between pH 7.2 and 4.6 was also incubated to determine whether plant tissue alone, at 37°C., might cause a change in the pH. After 3 days at 37°C. the pH determinations on the broth in these tubes showed that no changes had occurred.

Experiment 5.—The media were incubated for 3 days to insure sterility. 24 hour blood broth cultures of three strains of hemolytic streptococci and five strains of non-hemolytic streptococci were used for seeding. In each instance the tip of a platinum wire was inserted the distance of 1 cm. into the supernatant fluid of the blood broth culture and then carried into the test medium. The latter was then placed in the incubator at 37°C. The degree of clouding of the medium was noted at the hours given in Table V.

From the results shown in Table V it is evident that sterile unheated plant tissue has a marked growth-stimulating effect upon hemolytic and non-hemolytic streptococci. Heavy clouding of the potato broth cultures of the three strains of *Streptococcus hæmolyticus* and, to a less degree, of two of the five strains of non-hemolyzing streptococci occurred before there was macroscopic evidence of growth of these organisms in the plain broth without potato. In the remaining cultures of non-hemolytic streptococci at the end of 18 hours growth was much more abundant in the potato broth tubes than in the tubes of plain broth.

SUMMARY.

In previous papers it has been shown that unheated plant tissue, in the form of potato, contains the two factors necessary for the growth of organisms of the hemoglobinophilic group. Further studies (5) confirmed these findings and showed that yellow and white turnip, carrot, beet, parsnip, and sweet potato can replace blood in the cultivation of *Bacillus influenzæ*.

In the present paper it has been shown that vegetable tissues also greatly facilitate and stimulate the growth of other organisms entirely unrelated to *Bacillus influenzæ*. Three varieties of Gram-positive cocci have been used in the present study, pneumococcus, *Streptococcus hæmolyticus*, and *Streptococcus viridans*. With pneumococcus it has been previously shown that prompt and luxuriant growth will occur in broth containing unheated potato even though the seeding be so small that no growth whatever will occur with the same seeding in plain broth (5). In the present study it has been shown that even in dextrose broth this minimal inoculation is followed by a prolonged period of lag, whereas in potato broth this same inoculum serves to initiate immediate and rapid growth. When pneumococci are grown in potato broth not only is the period of lag abolished, but the sta-

tionary period of growth is extended and cell death is delayed. Moreover, in plant tissue medium the zone of hydrogen ion concentration within which growth of pneumococcus can be initiated is considerably extended beyond the acid and alkaline limits of the optimal range in ordinary bouillon.

It has been found also that the presence of unheated plant tissue in the media likewise stimulates growth of hemolytic and non-hemolytic streptococci.

In this investigation no attempt has been made to determine the exact nature of the substances in plant tissue upon which these properties depend. That they are not of the nature of readily fermentable carbohydrates, however, is made evident by the fact that no increased production of acid occurs in the pneumococcus culture when potato is present.

CONCLUSIONS.

1. The initiation of active growth in broth culture of pneumococcus, *Streptococcus hæmolyticus*, and *Streptococcus viridans* is accelerated by the presence of unheated plant tissue.

2. Cultures of pneumococcus in broth containing unheated plant tissue show a prolongation of the stationary phase of growth.

3. Death of the individual organisms in pneumococcus broth cultures containing unheated plant tissue does not proceed so rapidly as in cultures without plant tissue.

4. The zone of hydrogen ion concentration within which growth of pneumococcus in ordinary broth can be initiated is considerably extended, both on the acid and on the alkaline side, by the addition of unheated vegetable tissue to the media.

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THE SIZE OF THE HEART IN PNEUMONIA.

A TELEROENTGENOGRAPHIC STUDY, WITH OBSERVATIONS ON THE EFFECT OF DIGITALIS THERAPY.*

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Precise information concerning the behavior of the heart in pneumonia is essential for the rational management of the circulation in this disease. Whether, in the course of acute pneumonitis, the cardiovascular system is functionally impaired, is still a matter of controversy. Direct methods for the estimation of myocardial function are still wanting. But alterations in the size of the heart furnish evidence, although indirect, of changes in the physical state of the myocardium, and in particular of variations in the length of heart muscle fiber. Such changes, as will be pointed out, have been shown by investigation to be associated with alterations in the volume output of the ventricles; and on the maintenance of ventricular volume output depends, in large measure, the efficiency of the circulation as a whole.

The present investigation was designed to obtain, by the roentgenographic method, at relatively frequent intervals, accurate records of the size of the heart in patients ill with pneumonia. The cardiac silhouette was outlined and measured according to a special technic, and an attempt has been made to estimate the significance of the changes observed. The effect of digitalis on the size of the heart has also been studied.

LITERATURE.

It appears that Dietlen[1] is the only observer who has taken accurate records of the size of the heart in pneumonia throughout the course

* A preliminary report of this work was read before the American Society for Clinical Investigation, May 9, 1921.

1. Dietlen, H.: Orthodiagraphische Beobachtungen über Veränderungen der Herzgrösse bei Infektionskrankheiten, bei exsudativer Perikarditis und paroxysmaler Tachykardie, nebst Bemerkungen über das roentgenologische Verhalten bei Pneumonie. München. med. Wchnschr. 55: 2077, 1908.

of the disease. He made orthodiagrams of eleven cases of lobar pneumonia and found cardiac dilatation in four (36.4 per cent.). In two of these cases, the enlargement was slight and transitory; in the third case it was somewhat greater. No figures are given. Measurements of the area are recorded in the fourth case, and are now reprinted, since the changes observed are comparable to those to be reported in this paper.

On the sixth and seventh days of the disease, the patient was very ill. He was first afebrile on the eleventh day. Dietlen remarks that dilatation was coincident with the severest period of the illness—"the dangerous time before crisis."

TABLE 1.

Size of Heart Throughout Disease in Dietlen's Case.

Day of disease	2	3	5	6	7	11	12	14	19	21	24	25
Area, sq. cm.	123	123	133	135	134	123	122	122	122	122	117	116

MATERIAL AND METHODS.

Material.—Teleroentgenograms of the chest were made every second day, or at times as nearly approximating this interval as the condition of the patients warranted, of twenty-one cases of lobar pneumonia and eight cases of bronchopneumonia, during the acute stages of the disease and throughout convalescence in the hospital. Two hundred and eighty-four plates were taken and form the basis of this study. Three normal young men and one patient with fever due to acute bronchitis and pulmonary tuberculosis served as controls.

Method of Taking the Roentgenograms.—The exposures were made for one-half second, during the inspiratory phase of normal respiration, the subjects being in the recumbent position. The plates were at a distance of 2 meters from the anticathode of the roentgen-ray tube. The patient was instructed to breathe as quietly as possible and was unaware of the instant at which the picture was to be taken. In order to record graphically on the plate the phase of respiration in which the exposure was made, a lead strip was secured to the plateholder with adhesive plaster to indicate the neutral position of the vertically hanging lever of a Marey tambour, the lever being tipped with a solid

knob of lead. The tambour was connected by means of rubber tubing with a Politzer bag, held in position in the right axilla of the patient by a linen binder, secured by fastening its tails. During respiration, the

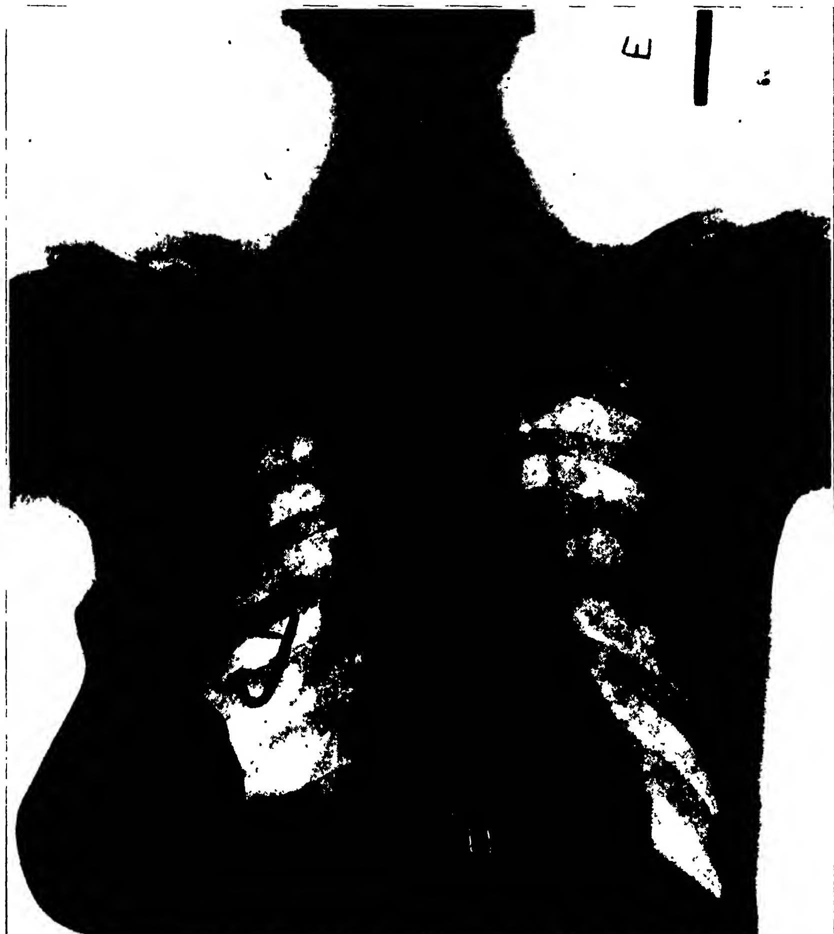

FIG. 1. Teleroentgenogram, showing method of taking the plates.

lever of the tambour swung on one side or the other of the neutral strip. The side to which the lever deviated during expiration was denoted by the letter *E* (Fig. 1). The pictures were obtained during inspiration in a majority of instances. Slight variations in the time of

exposure were disregarded, as it has been shown, employing a similar technic, that the difference in the size of the heart during normal inspiration and expiration is so small as to be negligible.[2]

In order to verify the accuracy of anteroposterior alignment, a thin lead strip was placed over the spinous processes of the vertebrae, and two acute angles of lead were secured anteriorly—one in the suprasternal notch, the other in the substernal angle. In later plates, the posterior lead strip was omitted, as the vertebral spines could be plainly seen in the negatives. Unclear or improperly aligned records were discarded.

A New Technic for Completing the Outline of the Cardiac Silhouette.—In the plates, the diastolic outline of the right and left borders and the midline were trached on paper. This made possible the measurement of transverse and long diameters. The angle of inclination of the heart—that is, the angle formed by the long diameter and a line drawn to the apex at right angles with the median line—was also recorded. Instead of completing the silhouette by arbitrarily joining the ends of the lines defining the borders, a simple geometric construction was adopted, which rendered the procedure entirely objective (Fig. 2). In Figure 2, the heavy lines *a d* and *b e* represent, respectively, right and left borders, traced from the roentgenogram. Using the distance *a b* as a radius and *a* as a center, an arc was drawn. With the same radius, but with *b* as a center, another arc was drawn, intersecting the first at the point *c*. With *c* as center and *a b* again as a radius, the points *a* and *b* were joined. The arc *d e* was constructed in like fashion, employing the radius *d e* and the point *f* as center. The area so delineated was measured with a planimeter.

It is evident that if the distances *a b* and *d e* are constant, the areas bounded by these lines and their corresponding arcs must likewise be constant in value. Variations in these distances, on the other hand, will be accompanied, in the same sense, by corresponding changes in the areas outlined. The subjective, arbitrary construction formerly employed may, therefore, properly be replaced by one which is objective, even though arbitrary in its variations.

2. Cohn, A. E.: An Investigation of the Size of the Heart in Soldiers by the Teleroentgen Method, Arch. Int. Med. **25:** 499 (May) 1920.

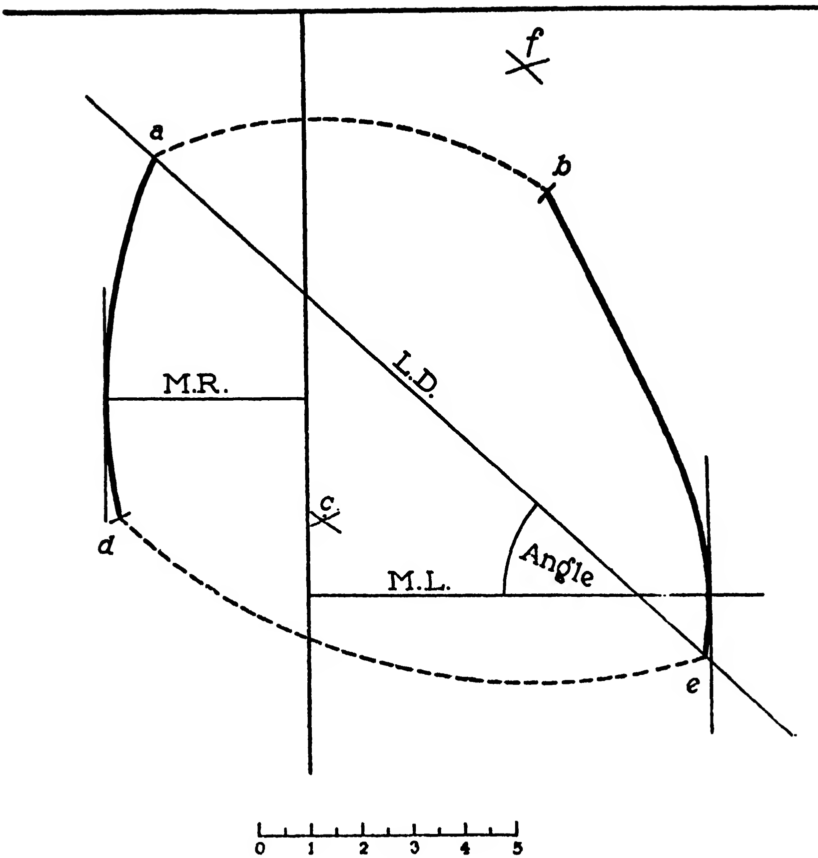

FIG. 2. Construction of the cardiac silhouette, showing area, diameters and angle.

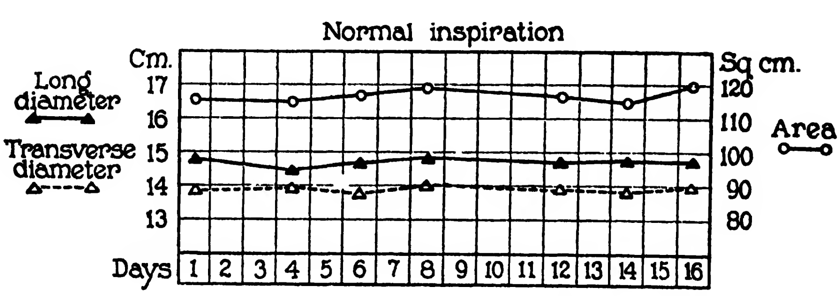

FIG. 3. Graph of cardiac measurements in a normal young man, G. S., aged 17. Seven plates were made during a sixteen day period.

CONTROL SERIES.

Variations in the cardiac measurements in the same person from day to day may be due either to the fact that significant changes in the size of the heart actually occur under ordinary conditions or to technical errors in the method of measurement. To obtain data on this point, roentgenograms were made, according to the technic described, of three normal young men who were workers in the laboratory. Plates were taken every second or third day until six or seven had been made (Table 2). Measurements of these roentgenograms

TABLE 2.

*Exposures Made During Normal Inspiration.**

Date	Horizontal Posture					
	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees
Feb. 18	5.4	7.9	13.3	14.0	111	35
Feb. 21	5.2	8.1	13.3	14.1	113	35
Feb. 23	5.7	7.7	13.4	14.1	113	37
Feb. 25	5.5	7.8	13.3	14.1	113	36
March 1	5.6	7.6	13.2	14.0	111	38
March 3	5.6	7.5	13.1	13.9	109	37
	Maximum Variations					
	0.5	0.5	0.3	0.2	4	3

* These roentgenograms were made of D. W. L., a man, aged 18 years, a normal person.

show that the maximum variation in transverse diameter was 0.6 cm., in long diameter 0.4 cm. and in area 8 sq. cm.[3]

In order to ascertain the effect of fever and tachycardia on the size of the heart, measurements of the heart were made in a patient with acute bronchitis and pulmonary tuberculosis, but without signs of consolidation in the lungs. The maximum variation in transverse diameter was 0.7 cm., in long diameter 0.2 cm. and in area 1 sq. cm. That fever and tachycardia per se are not necessarily associated with a

3. Measurements of *M R*, *M L* and the angle of inclination are included in the tables for purposes of reference, but will not be alluded to in the discussion.

change in the size of the heart was later abundantly confirmed by observations on cases of pneumonia in which no alteration was noted (Fig. 4).

It seemed reasonable, on the basis of these findings, to regard as a significant change in the outline of the heart a variation of 1 cm. or more in one of the diameters, or of 10 sq. cm. or more in area. Usually,

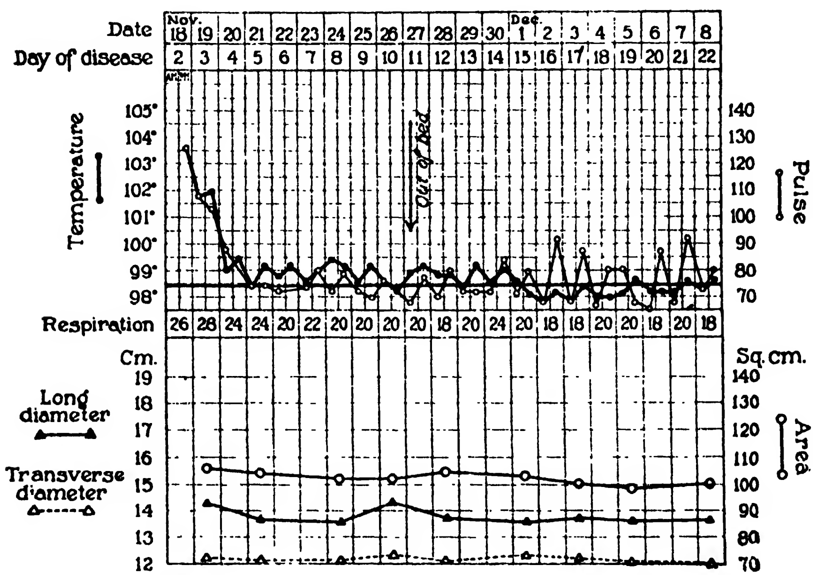

FIG. 4 (Case 24). Clinical chart and cardiac measurements of a woman, A. J., aged 27, who had bronchopneumonia of the right lower lobe; pneumococcus, group IV.

when a change was observed, it took place in all measurements, and was of a greater order of magnitude than the minimal criteria established.

RESULTS IN PNEUMONIA.

Lobar Pneumonia.—The twenty-one cases studied presented varying degrees of severity. Bacteriologic examination of the sputums showed the usual types of organisms (Table 3). The series is too small to permit of generalizations which take into account age and sex. Of the entire group, thirteen patients (61.9 per cent.) showed an increase

in the size of the heart during the course of the illness. Eleven of the patients did not, however, receive digitalis; to ten this drug was given, by mouth, in the form of digitan. Of the eleven patients not receiving digitalis, eight (72.7 per cent.) showed cardiac enlargement; of the

TABLE 3.

Summary of Twenty-One Cases of Lobar Pneumonia Grouped According to Pneumococcus Types.

	Number of Cases	Percentage
Type I	12	57.1
Type II	2	
Type IIA	1	15.1
Type III	1	5.1
Group IV	5	22.7
Type I—12 Cases		
No change in heart size	3	25
Change	9	75
Cases Not Receiving Digitalis—7		
No change in heart size	1	14.3
Change	6	85.7
Cases Receiving Digitalis—5		
No change in heart size	2	40
Change	3	60
Type II and IIA—3 Cases		
No change in heart size (1 received digitalis)	2	66.7
Change	1	33.3
Type III—1 Case		
Change—Digitalis given		
Group IV—5 Cases		
No change in heart size	3	60
Change	2	40
Cases Not Receiving Digitalis—2		
No change in heart size	1	50
Change	1	50
Cases Receiving Digitalis—3		
No change in heart size	2	66.7
Change	1	33.3

ten receiving this drug, five (50 per cent.) showed cardiac enlargement (Table 4). From these facts it appears that: (1) in about 62 per cent. of these cases of lobar pneumonia, cardiac dilatation occurred during the course of the disease; (2) cardiac dilatation occurred less frequently in the patients who received digitalis than in those who did not.

The order of magnitude of the change is of interest (Table 5). In the patients not receiving digitalis, the increase in transverse diameter was from 4.5 to 15.4 per cent. (an average of 9.3 per cent.); in long diameter from 4.4 to 13.5 per cent. (an average of 8.2 per cent.); in area from 11.6 to 27.1 per cent. (an average of 16.5 per cent.). In the digitalized patients who showed enlargement, the increase in trans-

TABLE 4.

Summary (284 Plates).

	Number of Cases	Percentage
Lobar Pneumonia—21 Cases		
No change in heart size	8	38.1
Change	13	61.9
(a) In all measurements 9		
(b) In long diameter and area only 4		
Cases Not Receiving Digitalis—11		
No change in heart size	3	27.3
Change	8	72.7
(a) In all measurements 6		
(b) In long diameter and area only 2		
Cases Receiving Digitalis—10		
No change in heart size	5	50
Change	5	50
(a) In all measurements 4		
(b) In long diameter and area only 1		
Bronchopneumonia—8 Cases		
No change in heart size	6	75
Change	2	25
(Change occurred in all measurements)		
Cases Not Receiving Digitalis or Strophanthin—6		
No change in heart size	4	66.7
Change	2	33.3
Cases Receiving Digitalis or Strophanthin—2		
No change in heart size	2	100
Change	0	

verse diameter was from 3.6 to 19.2 per cent. (an average of 11.0 per cent.); in long diameter from 2.6 to 19.7 per cent. (an average of 10.2 per cent.); in area from 11.1 to 37.3 per cent. (an average of 18.5 per cent.). The differences in the two groups are not striking. The averages in the second group are increased by the figures obtained from a patient with Type III pneumococcus infection involving the

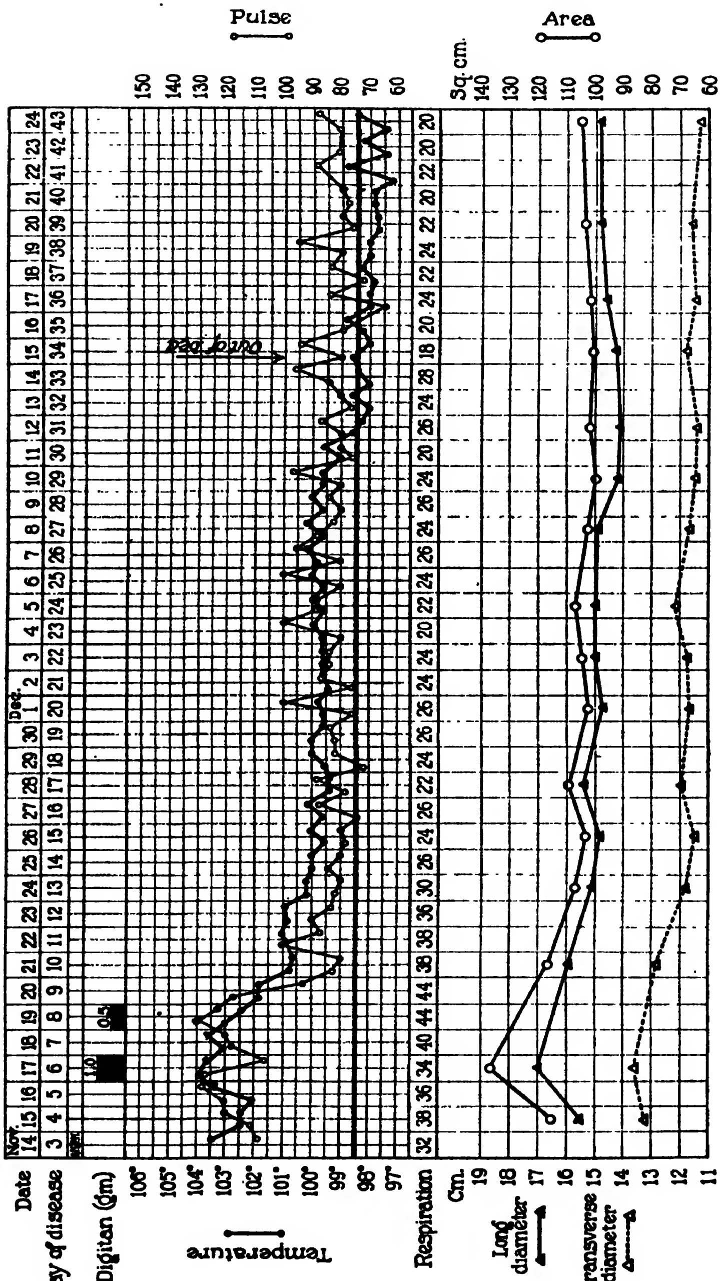

FIG. 5 (Case 15). Clinical chart and cardiac measurement of a man, aged 29, with pneumonia of the right upper, middle and lower lobes and of the left lower lobe; pneumococcus, Type III.

entire right lung and the left lower lobe. In this man, critically ill, the transverse diameter fell from 13.6 cm. at the height of the disease to 11.4 cm. during convalescence, the long diameter from 17.0 to 14.2 cm., the area from 136 sq. cm. to 99 sq. cm. (Figs. 5 and 6). The correspondence in this case between the size of the heart and the

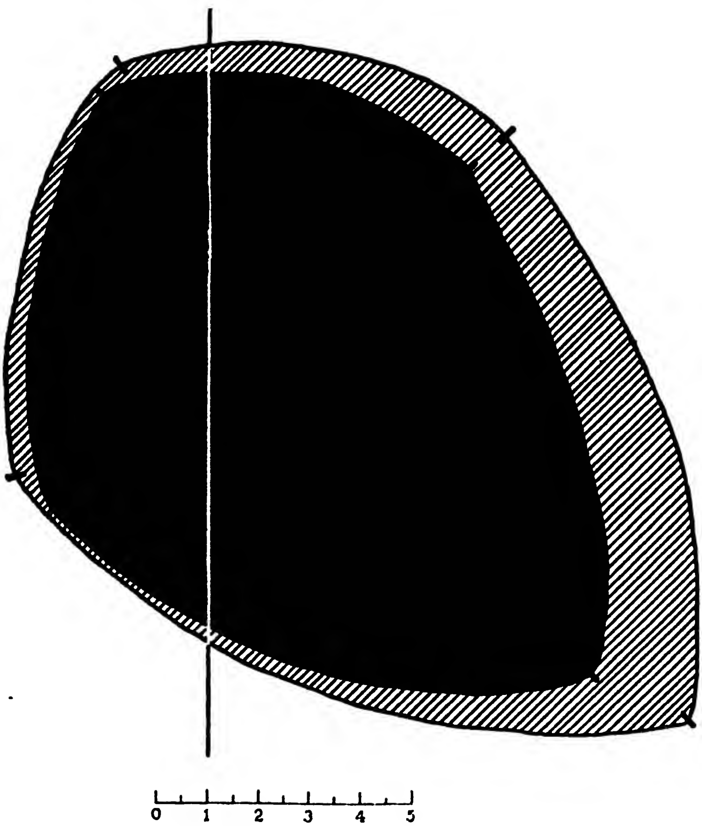

FIG. 6 (Case 15). Silhouettes showing extremes of variation in heart size.

clinical condition is striking. Digitalis was not administered until the sixth day of illness, at a time when the patient's life hung in the balance. The temperature from this day on fell by lysis and the heart decreased in size, reaching a constant level on the twentieth day of disease.

TABLE 5.

Summary of Twenty-One Cases of Lobar Pneumonia and Eight Cases of Bronchopneumonia, Showing Relation of Changes in Heart Size to Digitalis Therapy.

Case No.; Name; Age	Bacteriology	Lobes Involved	Heart Size	Maximal Percentage Change			Digitalis or Strophanthin	Total Amount Given	Day of Disease Drug Begun	Serum
				Area	Transverse Diameter	Long Diameter				
1. E. M. 52	Pneumococcus I Bacillus influenzae	L. L.	+	16.2	9.0	9.7	0	......	..	+
2. M. F. 29	Pneumococcus I	L. L.	+	27.1	12.2	13.5	0	......	..	+
3. S. P. 36	Pneumococcus I Bacillus influenzae	L. L.	+	19.5	7.1	10.5	0	......	..	+
4. H. D. 39	Pneumococcus I	L. L.	+	11.6	4.5	6.5	0	......	..	+
5. P. K. 19	Pneumococcus I Bacillus influenzae	L. L.	+	12.3	5.8	4.2	0	......	..	+
6. L. M. 21	Pneumococcus I Bacillus influenzae	R. L., L. L.	+	12.5	12.3	7.1	0	......	..	+
7. F. T. 29	Pneumococcus IV Bacillus influenzae	R. L.	+	15.0	8.5	11.4	0	......	..	..
8. T. M. 47	Pneumococcus IIA	R. L.	+	18.5	15.4	4.4	0	......	..	..
9. J. K. 18	Pneumococcus II	R. L., L. L.	0	0	0	0	0	......	..	..
10. J. S. 28	Pneumococcus IV	L. L.	0	0	0	0	0	......	..	..
11. S. B. 13	Pneumococcus I Bacillus influenzae	R. U.	0	0	0	0	0	......	..	0
12. J. C. 30	Pneumococcus I	R. L.	+	11.1	3.6	2.6	+	0.6 gm.	5	+
13. P. O'M. 25	Pneumococcus I	R. U., R. M., R. L.	+	18.4	8.9	8.8	+	0.8 gm.	4	+

14. E. D. 39	Pneumococcus I	R. U., L. L.	+	14.7	13.0	10.4	+	1.5 gm.	4	+
15. A. T. 29	Pneumococcus III	R. U., R. L., R. M., L. L.	+	37.3	19.2	19.7	+	1.5 gm.	6	..
16. F. L. 32	Pneumococcus IV	L. L.	+	11.2	10.2	9.7	+	1.5 gm.	4	..
17. V. P. 26	Pneumococcus I	R. L.	0	0	0	0	+	1.5 gm.	5	+
18. M. L. 34	Pneumococcus I Bacillus influenzae	L. L.	0	0	0	0	+	0.9 gm.	2	+
19. L. W. 16	Pneumococcus II Bacillus influenzae	R. L.	0	0	0	0	+	1.8 gm.	2	..
20. A. R. 37	Pneumococcus IV	R. U.	0	0	0	0	+	0.9 gm.	6	..
21. R. L. 34	Pneumococcus IV Bacillus influenzae	L. L.	0	0	0	0	+	1.0 gm.	2	..
				Bronchopneumonia						
22. M. Q. 39	Pneumococcus IV Bacillus influenzae	Dif. bilat.	+	17.1	3.7	5.2	0	......	..	..
23. E. S. 55	Pneumococcus IV	L. L.	+	29.8	12.0	20.1	0	......	..	..
24. A. J. 27	Pneumococcus IV	R. L.	0	0	0	0	0	......	..	..
25. G. B. 13	Streptococcus hemolyticus	R. L.	0	0	0	0	0	......	..	..
26. C. G.* 35	Streptococcus hemolyticus Bacillus influenzae	Dif. bilat.	—	—	—	—	0	......	..	..
27. M. K. 19	Pneumococcus IV	R. L.	0	0	0	0	Dig.	1.0 gm.	6	..
28. A. D. P.† 34	Staphylococcus aureus Bacillus influenzae	R. L.	0	0	0	0	Stroph.	0.7 mg.	8	..
29. G. E.‡ 7	Pneumococcus IV Bacillus influenzae	Dif. bilat.	0	0	0	0	Stroph.	0.5 mg.	21	..

* Died. † Patient had mitral stenosis. ‡ Died; strophanthin given late.

As a rule, the increase in heart size was gradual, often reaching its maximum at the height of the disease, but sometimes after the subsidence of fever. The return to a constant level was also gradual, at times lagging behind the fall in temperature and pulse rate (Figs. 7 and 8). In a number of cases, there was marked postfebrile decrease in size, the heart at this time being smaller than during later convalescence (Figs. 9 and 10). In two instances, one patient receiving digitalis (Case 16), the other (Case 7) not receiving this drug, the heart was distinctly larger during convalescence than during the height of the illness. In both cases there was pronounced bradycardia during the convalescent period.

In the thirteen patients showing increase in the size of the heart, the change was observed in all measurements in nine; in long diameter and area only, in four. The transverse diameter was accordingly the least reliable index of variation. The greatest percentage variation occurred in the area. It is impossible to attempt to determine from the tracings which chamber or chambers of the heart participated in the enlargement. For the method employed measures only a portion of the heart, in a single plane.

Bronchopneumonia.—Eight cases were studied (Tables 4 and 5). Of these, a change in the size of the cardiac silhouette occurred in two (25 per cent.), and was observed in all measurements. In one patient with diffuse, bilateral, streptococcus pneumonia, of whom plates were made until three days before death, there was seen a progressive diminution in the size of the heart (Case 26). No observations were made during the last three days. He received no digitalis.

Of the eight cases, one, a boy of 19 years, received digitalis, beginning on the sixth day of the disease; another, who had mitral stenosis, received 0.7 mg. of strophanthin intravenously on the eighth day of illness. Neither of these patients showed a change in the size of the heart. The drugs were given late in the course of the pneumonia so that their influence on the behavior of the heart is a matter of doubt.

In brief, it is apparent that cardiac dilatation is less frequently encountered in bronchopneumonia (25 per cent.) than in lobar pneumonia (61.9 per cent.). When it occurs, as will be seen from Table 5 and from Figures 7 and 8, the change is comparable in degree and character to that seen in the latter condition. Evidence of the in-

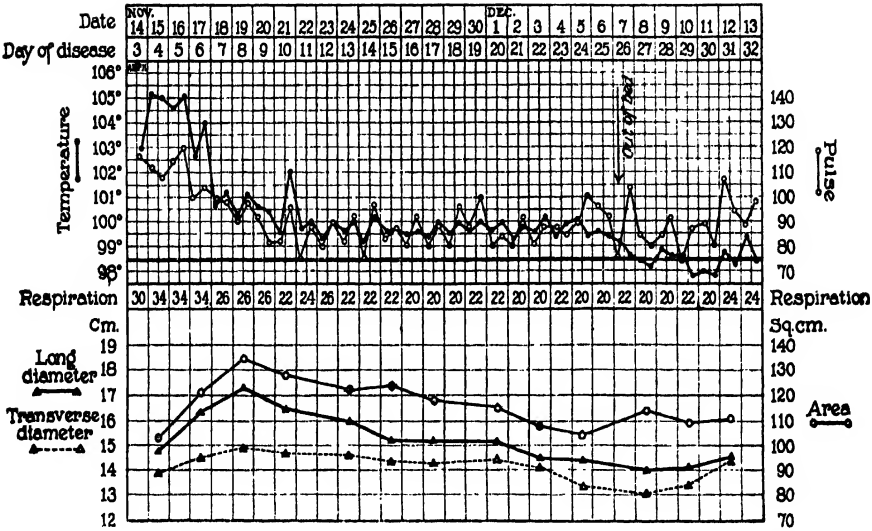

Fig. 7 (Case 23). Clinical chart and cardiac measurements of a woman, aged 55, with bronchopneumonia of the left lower lobe; pneumococcus, Group IV.

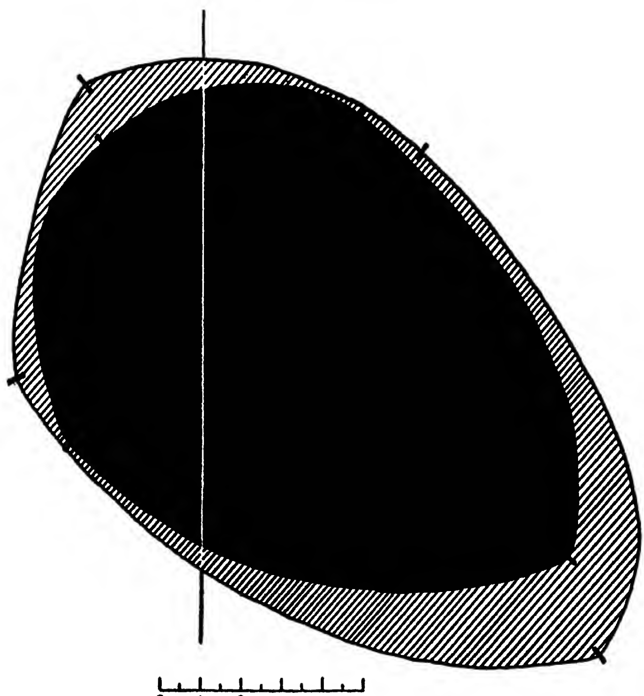

Fig. 8 (Case 23). Silhouettes showing extremes of variation in heart size.

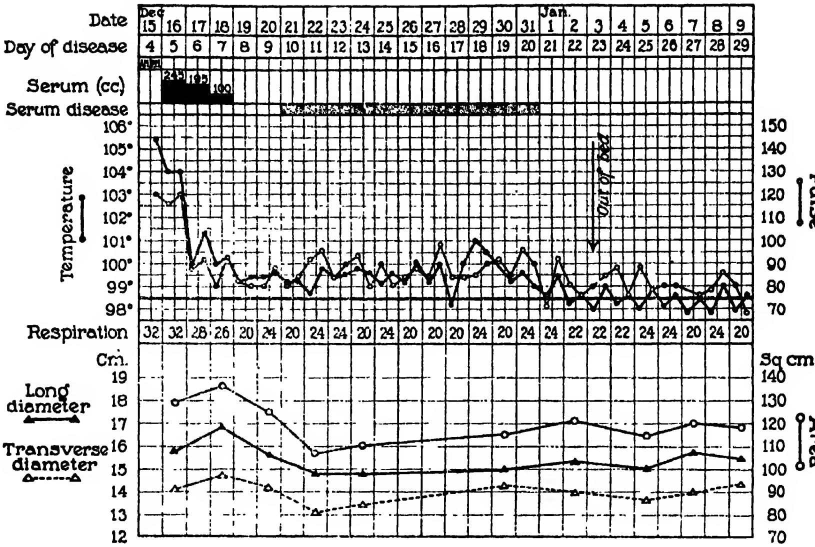

Fig. 9 (Case 2). Clinical chart and cardiac measurements of a woman, aged 29, with lobar pneumonia of the left lower lobe; pneumococcus, Type I.

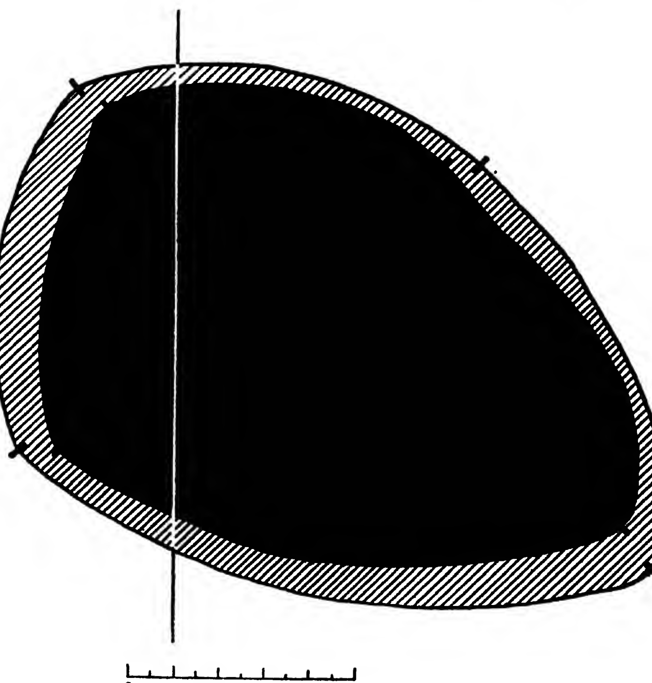

Fig. 10 (Case 2). Silhouettes showing extremes of variation in heart size.

fluence of digitalis is scant, but it tends to support the view that when the drug is given, cardiac dilatation is less likely to occur.

DISCUSSION.

Cardiac Dilatation.—The heart is able to adapt itself in remarkable fashion to variations in the demands made on it in the form of mechanical work. The mechanism of this adaptation has been made the subject of particular study by Starling,[4] employing the heart-lung preparation. He found that any increase in the work of the heart, however caused, whether by a rise in the arterial pressure or by a more rapid venous inflow, is attended by a corresponding increase in the volume of the ventricles. And, within physiologic limits, the larger the volume of the heart, the greater is the energy of its contractions as measured by systolic output. The law of the heart is thus the same as the law of muscular tissue in general, that "the energy of contraction, however measured, is a function of the length of the muscle fibre." Up to a physiologic optimum, cardiac dilatation is an adaptive, compensatory mechanism favorable to the maintenance of an adequate circulatation. In a healthy person, to accomplish this adaptation may be only a temporary necessity; for the circulation improves as a result of augmented cardiac efficiency, the cardiac tone itself increases, and the heart gradually returns to a normal volume even though doing increased work. But, when the heart is fatigued or diseased, the secondary improvement fails to appear, and the myocardium remains dilated over the period of increased work. If the work is prolonged, dilatation may become permanent, and may even exceed the optimum length of muscle fiber. If this occurs, the muscle must contract at so great a mechanical disadvantage that heart failure ensues.

Anatomic lesions in the heart in pneumonia are, according to a majority of investigators, infrequent and, relatively speaking, insignificant.[5] In the pneumonias associated with influenza in soldiers,

4. Starling, E. H.: The Linacre Lecture on the Law of the Heart, Longmans, Green & Co., London, 1918.

5. Cohn, A. E., and Jamieson, R. A.: The Action of Digitalis in Pneumonia, J. Exper. M. 25: 65, 1917. (This article, on p. 73, contains a review of the literature.)

Stone,[6] however, reports parenchymatous, fatty and hyaline degeneration or evidence of inflammatory reaction in the heart muscle in 79.3 per cent. of the microscopic sections from thirty-four necropsies of cases of lobar pneumonia and in 59.4 per cent. of the sections from thirty-seven necropsies of cases of bronchopneumonia. It is of interest to recall, in this connection, the greater frequency, in the present study, of cardiac dilatation in lobar pneumonia (61.9 per cent.) than in bronchopneumonia (25 per cent.). Aside from structural change, there are present in pneumonia at least three conditions which are concerned in throwing an added burden of work on the heart. These are: (1) impairment of the circulation in the pneumonic lung; (2) toxemia, and (3) anoxemia.

(1) Impairment of the Circulation in the Lung: In a study of injected lungs from patients dying of lobar pneumonia, Kline and Winternitz[7] and, more recently, Gross[8] have demonstrated varying degrees of vascular obliteration, depending in extent on the stage of pulmonary consolidation. In a lung described by Gross, in which the two upper lobes were consolidated, the lower lobe showed tremendous dilatation of the vessels with massive injection. The capillaries appeared to be two or three times their normal caliber, and the entire lobe presented a striking picture of compensatory arterial dilatation. In the upper lobe, there was some preservation of the circulation, but the injected vessels were narrowed. The middle lobe showed general lack of injection; only several large, compressed branches were apparent, and these ended abruptly. Thus, when exudation is at its height, there is almost complete vascular obliteration. With the arterial bed in the lung markedly diminished in area, the pressure in the pulmonary artery is increased, and the right ventricle is obliged to contract against a heightened resistance.[9] In bronchopneumonia this factor is probably of less significance than in lobar pneumonia.

6. Stone, W. J.: The Heart Muscle Changes in Pneumonia, with Remarks on Digitalis Therapy, Am. J. M. Sc. 163: 659, 1922.

7. Kline, B. S., and Winternitz, M. C.; Studies upon Experimental Pneumonia in Rabbits. VIII. Intra Vitam Staining in Experimental Pneumonia and the Circulation in the Pneumonic Lung, J. Exper. M. 21: 311, 1915.

8. Gross, L.: Preliminary Report on the Reconstruction of the Circulation of the Liver, Placenta and Lung in Health and Disease, Canad. M. A. J. 9: 632, 1919.

9. Wiggers, C. J.: Circulatory Failure, J. A. M. A. 70: 508 (Feb. 23) 1918.

(2) Toxemia: The heart muscle in pneumonia is fed by blood containing bacterial toxins and perhaps also products of autolysis yielded by the exudate in the lung alveoli. Pertinent experimental evidence on the effect of these poisons on the heart is afforded by the work, on dogs, of Newburgh and Porter.[10] They found that the heart muscle is not functionally impaired in pneumonia, since the pneumonic ventricle beats normally as soon as it is fed with normal blood; that pneumonic blood, suddenly fed to normal heart muscle, lowers its efficiency, lessening the duration and the force of contraction; but that the heart muscle in pneumonia, exposed gradually to the action of the poison, largely adjusts itself to the poisoned food. Clinical observations lend support to these findings.

(3) Anoxemia: High arterial unsaturation, with its accompanying clinical manifestation, cyanosis, is a frequent occurrence in both lobar and bronchopneumonia, due, in large measure, according to Stadie,[11] to an impairment in the function of the respiratory surface of the lungs. The greater the lung involvement, the greater is the anoxemia. Especially is this so when the pneumonic process extends throughout the lungs, as is the case when there are many patches of bronchopneumonia, with accompanying bronchitis and edema. That the circulation as a whole may suffer from oxygen want is evident from Stadie's observation that, after administration of oxygen in 60 per cent. concentration in a closed chamber, to a moribund and pulseless patient, the heart rate fell from 160 to 120, the pulse returned to the radial artery, the color became pink, and there was remarkable clinical improvement.[12] Other, less striking observations support this view. No records of the size of the heart were made in his cases.

It seems , then, that for the reasons given, and perhaps others, the heart may be called on to do more than its usual quota of mechanical work in pneumonia, and that, in certain instances, it responds to the increased demands made on it by dilatation, with resultant lengthen-

10. Newburgh, L. H., and Porter, W. T.: The Heart Muscle in Pneumonia, J. Exper. M. 22: 123, 1915.

11. Stadie, W. C.: The Oxygen of the Arterial and Venous Blood in Pneumonia and Its Relation to Cyanosis, J. Exper. M. 30: 215, 1919.

12. Stadie, W. C.: The Treatment of Anoxemia in Pneumonia in an Oxygen Chamber, J. Exper. M. 25: 337, 1922.

ing of its muscle fibers. Whether the physiologic optimum of lengthening is ever exceeded, remains an open question. Certainly the signs of heart failure, in the ordinary sense, are conspicuous by their absence. The venous pressure, taken by the method of Hooker, was, moreover, in a number of our cases, within normal limits. The mechanism of adaptation, as has been pointed out, is a gradual one, sometimes reaching its maximum after the height of the disease has been passed. The return of the heart to a smaller and constant volume is likewise a gradual process, and, in that sense, may be regarded as indicating recovery from a functional injury.

The Therapeutic Use of Digitalis.—Clinicians are agreed that when auricular fibrillation or flutter occurs in the course of pneumonia, the prompt administration of digitalis in adequate doses exerts not merely a beneficial effect but is at times responsible for the saving of life. In the presence of normal (sinus) rhythm, there is still doubt as to its value. Cohn and Jamieson[5] have pointed out that in patients with pneumonia the usual digitalis effects on auriculoventricular conduction and on the T-wave of the electrocardiogram may be obtained; and that whatever beneficial action the drug has on the function of the normally beating, nonfebrile heart may also be expected from its use in pneumonia.

Another consideration, however, leads to the suggestion that digitalis may be a rationally useful therapeutic measure. Cohn and I [13] have shown, in dogs, that digitalis, in amounts comparable to the therapeutic dose in man, increases the contractile power of the ventricular muscle, and by so doing augments systolic volume output. At a time when the increase in output occurs, there are usually characteristic changes in the T-wave of the electrocardiogram.

From the facts presented in this paper, it is evident that the heart muscle in pneumonia may attempt, by calling on a reserve mechanism, dilatation, to augment systolic output. In a relatively large number of the patients who received digitalis, the heart did not increase in size. It, therefore, seems reasonable to infer that when the ventricle is stimulated to increased force of contraction by digitalis, the compensatory mechanism of dilatation, in some instances at least, is not called

13. Cohn, A. E., and Levy, R. L.: The Effect of Therapeutic Doses of Digitalis on the Contraction of Heart Muscle, Proc. Soc. Exper. Biol. & Med. **17:** 160, 1920.

into play. To that extent, the reserve force of the heart is not encroached on. It is, therefore, logical to suggest the use of early and adequate digitalis therapy in pneumonia, in the belief that it may exert a beneficial effect not only in the presence of auricular fibrillation and flutter, but also when the normal rhythm prevails.

SUMMARY.

1. A teleroentgenographic study was made of twenty-one cases of lobar pneumonia and eight cases of bronchopneumonia, employing a special technic in making the plates and a new method in completing the outline of the cardiac silhouette.

2. In lobar pneumonia, cardiac dilatation occurred in 61.9 per cent. of the cases. Dilatation was less frequently observed in patients who received digitalis (50 per cent.) than in those to whom the drug was not given (72.7 per cent.).

3. In bronchopneumonia, cardiac dilatation was less frequent than in lobar pneumonia, having been observed in 25 per cent. of the cases. The two patients in this group who received digitalis showed no dilatation.

4. The heart muscle in pneumonia may attempt, by lengthening its muscle fibers, to augment systolic output. Digitalis, in therapeutic doses, increases the contractile power of the ventricles. On the basis of these facts, early and adequate digitalis therapy is suggested in pneumonia, in the belief that the drug may exert a beneficial effect, not only in the presence of auricular fibrillation and flutter, but also when the normal rhythm prevails.

To the painstaking efforts of Dr. W. D. Witherbee and Mrs. E. Jackson is due the accuracy with which the roentgenograms were made.

TABLE 6.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data			
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Serum, C.c.	Serum, Disease	Maximum Temperature	Maximum Pulse
1/ 2/20	5	4151	2.8	9.2	12.0	13.3	89	25	190	..	104.5	128
1/ 5/20	8	4158	2.9	9.0	11.9	13.5	93	36	...	..	100.1	80
1/ 7/20	10	4164	2.4	9.3	11.7	12.6	84	29	...	..	100.1	80
1/ 9/20	12	4171	2.9	8.1	11.0	12.3	81	32	...	..	100.0	84
1/14/20	17	4184	3.2	8.3	11.5	12.4	82	31	...	..	99.0	86
1/16/20	19	4193	3.2	8.3	11.5	12.7	83	32	...	..	99.6	84
1/19/20	22	4204	3.3	8.7	12.0	13.3	88	33	...	..	99.6	84
1/21/20	24	4215	3.5	7.8	11.3	12.7	80	33	...	..	99.3	96
1/23/20	26	4227	3.6	8.4	12.0	13.0	86	31	...	+	99.3	80
1/26/20	29	4237	3.3	8.4	11.7	13.0	82	33	...	+	99.4	85
2/ 8/21	Returned	4997	4.0	8.4	12.8	13.1	85	28	...	..	....	86

* Case 1, E. M., a man, aged 52 years, had pneumonia of the left lower lobe. Pneumococcus, Type I, and B. influenzae were present. The patient recovered.

TABLE 7.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data			
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle. Degrees	Serum, C.c.	Serum, Disease	Maximum Temperature	Maximum Pulse
12/15/19	4	....	...	...	....	....	...	..	...	..	105.4	124
12/16/19	5	4104	4.5	9.6	14.1	15.8	129	40	245	..	105.2	128
12/17/19	6	....	...	....	....	....	...	..	195	..	103.0	108
12/18/19	7	4113	4.0	10.7	14.7	16.8	136	40	100	..	101.5	100
12/20/19	9	4120	3.6	10.6	14.2	15.6	125	39	...	..	99.6	88
12/21/19	10	....	...	....	....	....	...	..	...	+	99.2	88
12/22/19	11	4122	2.9	10.2	13.1	14.8	107	40	...	+	99.7	96
12/24/19	13	4132	3.0	10.4	13.4	14.8	110	38	...	+	99.7	93
12/30/19	19	4140	3.0	11.2	14.2	15.0	115	33	...	+	99.8	92
1/ 2/20	22	4149	4.2	9.7	13.9	15.3	120	37	...	..	99.0	90
1/ 3/20	23	....	...	....	....	....	...	..	...	..	99.6	84
1/ 5/20	25	4155	3.9	9.8	13.7	15.0	115	39	...	..	99.6	88
1/ 7/20	27	4163	3.5	10.4	13.9	15.7	120	41	...	..	98.4	88
1/ 9/20	29	4168	4.4	9.9	14.3	15.4	118	38	...	..	98.6	90
2/ 4/21	Returned	4978	5.0	8.3	13.3	14.3	113	41	...	..	...	..

* Case 2, M. F., a woman, aged 29 years, had pneumonia of the left lower lobe. Pneumococcus, Type I, was present. The patient recovered.

TABLE 8.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data			
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Serum, C.c.	Serum, Disease	Maximum Temperature	Maximum Pulse
11/25/19	4	....	...	....	....	....	...	..	...	..	103.8	120
11/26/19	5	4008	4.5	11.3	15.8	16.0	145	32	300	..	103.8	120
11/27/19	6	....	...	....	....	....	...	..	195	..	102.8	108
11/28/19	7	4014	4.7	10.7	15.4	16.0	141	38	...	..	100.2	80
12/ 1/19	10	4028	3.8	12.7	16.5	16.7	147	33	...	..	99.2	76
12/ 2/19	11	....	...	....	....	....	...	..	...	+	99.2	78
12/ 3/19	12	4042	4.1	11.3	15.4	15.1	123	32	...	+	99.6	99
12/ 5/19	14	4061	4.1	11.4	15.6	14.8	124	34	...	+	99.0	78
12/ 8/19	17	4069	3.4	11.9	15.3	14.8	124	29	...	+	99.4	72
12/10/19	19	4083	4.5	11.1	15.6	15.7	...	28	...	+	99.4	86
12/12/19	21	4090	4.4	11.1	15.5	15.3	126	34	...	..	99.2	78
12/15/19	24	4100	4.2	11.3	15.5	15.8	132	34	...	..	99.0	78
2/ 2/21	Returned	4963	5.6	9.0	14.6	15.5	138	44	...	..	....	..

* Case 3, S. P., a man, aged 36 years, had pneumonia of the left lower lobe. Pneumococcus, Type I, and B. influenzae were present. The patient recovered.

TABLE 9.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data			
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Serum, C.c.	Serum, Disease	Maximum Temperature	Maximum Pulse
4/28/21	4	....	...	....	....	....	...	..	90	..	106.0	115
4/29/21	5	5423	5.0	11.1	16.1	15.5	144	22	200	..	106.8	122
4/30/21	6	....	...	....	....	....	...	..	100	..	101.6	85
5/ 2/21	8	5433	5.1	11.1	16.2	16.2	144	24	...	..	99.5	80
5/ 4/21	10	5453	5.6	9.9	15.5	15.3	131	26	...	..	99.0	72
5/ 6/21	12	5467	5.5	10.1	15.6	15.2	130	26	...	..	98.6	76
5/ 9/21	15	5476	5.4	10.3	15.7	15.3	129	25	...	+	99.4	68
5/11 21	17	5483	5.2	10.6	15.8	15.3	132	23	...	+	98.8	72
5/13/21	19	5494	5.5	10.1	15.6	15.4	131	24	...	..	99.2	74
5/16/21	22	5500	4.6	11.1	15.7	15.4	133	22	...	..	98.8	68
5/18/21	24	5512	5.3	10.5	15.8	15.3	129	21	...	..	99.4	68
5/20/21	26	5518	5.4	10.4	15.8	15.3	131	23	...	..	99.2	72

* Case 4, H. D., a man, aged 39 years, had pneumonia of the left lower lobe. Pneumococcus, Type I, was present. The patient recovered.

TABLE 10.

*Findings in a Case of Lobar Pneumonia.**

Date.	Day of Disease	Roentgenographic Data							Clinical Data			
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Serum, C.c.	Serum, Disease	Maximum Temperature	Maximum Pulse
11/24/19	4	....	...	...	....	....	...	..	...	..	105.0	134
11/25/19	5	4000	5.0	8.7	13.7	14.3	114	37	190	..	105.6	132
11/26/19	6	4009	4.7	9.2	13.9	14.4	112	35	195	..	106.0	130
11/28/19	8	4015	4.7	9.5	14.2	14.8	125	39	...	..	99.4	68
11/30/19	10	....	...	....	....	....	...	..	...	+	99.0	60
12/ 1/19	11	4029	4.6	10.0	14.6	14.8	127	35	...	..	99.1	54
12/ 3/19	13	4040	4.8	9.3	14.1	14.5	120	36	...	..	99.6	72
12/ 5/19	15	4060	3.9	10.1	14.0	13.7	114	30	...	..	99.4	74
12/ 6/19	16	....	...	....	....	....	...	..	...	+	99.0	68
12/ 8/19	18	4068	4.9	8.9	13.8	14.2	113	34	...	..	99.0	70
12/10/19	20	4082	4.4	9.8	14.2	14.7	120	33	...	..	99.1	72
12/12/19	22	4091	5.1	9.2	14.3	15.0	128	38	...	..	99.2	64
12/15/19	25	4099	4.3	10.2	14.5	14.7	125	33	...	..	99.4	72
2/ 7/21	Returned	4991	4.9	9.1	14.0	14.1	115	36	...	..	....	..

* Case 5, P. K., a man, aged 19 years, had pneumonia of the left lower lobe. Pneumococcus, Type I, and B. influenzae were present. The patient recovered.

TABLE 11.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data			
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Serum, C.c.	Serum, Disease	Maximum Temperature	Maximum Pulse
12/ 3/19	4	....	...	....	....	....	...	..	75	..	104.8	124
12/ 4/19	5	4050	5.3	10.4	15.7	16.5	144	36	195	..	105.0	112
12/ 5/19	6	....	...	....	....	....	...	..	290	..	103.0	102
12/ 6/19	7	...	...	....	....	....	...	..	265	..	101.6	96
12/ 8/19	9	4071	4.0	11.3	15.3	16.8	132	32	...	+	100.8	86
12/10/19	11	4084	4.9	10.1	15.0	15.9	131	36	...	+	101.6	88
12/12/19	13	4095	4.3	10.6	14.9	15.4	128	31	...	+	102.4	100
12/15/19	16	4101	4.4	10.8	15.2	16.0	131	32	...	..	99.6	90
12/17/19	18	4110	4.9	10.3	15.2	16.4	136	34	...	..	99.4	76
12/19/19	20	4116	4.9	10.0	14.9	15.6	129	34	...	..	99.6	78
12/22/19	23	4123	4.1	10.8	14.9	16.1	130	35	...	..	99.2	88
12/24/19	25	4135	3.9	11.1	15.0	15.9	133	33	...	..	99.6	88
12/30/19	31	4143	4.8	10.2	15.0	16.4	138	36	...	..	99.5	88
2/ 7/21	Returned	4990	4.8	10.5	15.3	15.6	141	34	...	..	....	..

* A case 6, L. M., a man, aged 21 years, had pneumonia of the right and left lower lobes. Pneumococcus, Type I, and B. influenzae were present. The patient recovered.

TABLE 12.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data	
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Maximum Temperature	Maximum Pulse
3/ 9/21	6	....	...	...	....	....	...	..	103.9	100
3/10/21	7	5155	4.0	9.7	13.7	14.1	109	36	104.6	92
3/12/21	9	5167	4.6	8.5	13.1	13.9	103	40	100.0	88
3/14/21	11	5169	5.7	7.5	13.2	13.4	101	38	100.0	72
3/16/21	13	5185	5.4	7.5	12.9	13.1	100	39	100.1	72
3/18/21	15	5196	5.6	7.4	13.0	13.3	101	38	99.6	72
3/21/21	18	5211	5.5	8.0	13.5	13.7	106	38	99.4	84
3/23 21	20	5230	5.5	8.5	14.0	14.1	110	35	99.4	72
3/25/21	22	5249	5.0	8.9	13.9	14.1	112	34	99.6	84
3/28/21	25	5262	5.5	8.4	13.9	14.6	115	33	99.2	76
3/30/21	27	5275	4.8	9.0	13.8	14.3	113	33	99.4	72

* Case 7, F. T., a man, aged 29 years, had pneumonia of the right lower lobe. Pneumococcus, Group IV, and B. influenzae were present. The patient recovered

TABLE 13.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data	
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Maximum Temperature	Maximum Pulse
12/ 4/19	5	....	...	...	....	....	...	..	103.8	98
12/ 5/19	6	4059	4.2	11.6	15.8	16.5	134	33	103.0	96
12/ 8/19	9	4070	3.7	11.0	14.7	16.8	127	37	99.5	72
12/10/19	11	4081	4.4	9.1	13.5	15.9	117	39	99.1	83
12/12/19	13	4092	5.0	8.7	13.7	15.8	113	37	99.3	80
12/15/19	16	4098	5.2	8.9	14.1	15.5	114	35	99.0	78
12/17/19	18	4109	4.8	9.5	14.3	15.9	123	33	99.0	84
12/18/19	19	....	...	....	....	....	...	..	99.0	78
12/19/19	20	4118	4.5	10.0	14.6	15.8	119	28	99.0	84
12/22/19	23	4125	4.5	9.4	13.9	15.4	120	34	90.0	80
12/24/19	25	4134	3.8	10.5	14.3	15.6	122	32	99.2	82
12/30/19	31	4144	3.8	10.8	14.7	15.7	121	27	99.2	80

* Case 8, T. M., a man, aged 47 years, had pneumonia of the right lower lobe. Pneumococcus, Type IIA, was present. The patient recovered.

TABLE 14.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data	
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Maximum Temperature	Maximum Pulse
4/12/21	6	....	...	....	....	....	...	..	104.1	148
4/13/21	7	5338	3.4	12.1	15.5	15.6	127	26	102.9	102
4/18/21	12	5336	3.7	12.0	15.7	15.9	130	25	99.9	72
4/20/21	14	5382	4.4	10.8	15.2	15.6	120	29	99.4	82
4/22/21	16	5391	4.5	11.1	15.6	15.7	126	26	99.9	80
4/25/21	18	5399	5.3	10.3	15.6	15.8	123	28	99.6	84
4/27/21	20	5406	5.5	10.1	15.6	16.0	125	30	99.2	82
4/29/21	22	5417	5.2	9.9	15.1	15.5	118	28	99.4	82
5/ 2/21	26	5431	5.6	9.9	15.5	15.5	122	26	100.0	88
5/ 4/21	28	5451	5.5	9.9	15.4	15.8	128	29	99.6	86
5/ 6/21	30	5464	5.5	9.8	15.3	15.6	124	32	100.0	80
5/ 9/21	33	5474	5.4	10.5	15.9	16.0	130	30	100.0	80

* Case 9, J. K., a youth, aged 18 years, had pneumonia of the right and left lower lobes. Pneumococcus, Type II, was present. The patient recovered.

TABLE 15.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data	
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Maximum Temperature	Maximum Pulse
3/28/21	5	....	...	...	....	....	...	..	105.0	126
3/29/21	6	5270	4.7	8.6	13.3	14.4	105	39	104.4	108
3/31/21	8	5280	4.6	8.8	13.4	14.3	106	40	99.2	72
4/ 2/21	10	5294	3.9	9.7	13.4	14.3	104	36	99.2	80
4/ 5/21	13	5307	4.6	8.3	12.9	13.7	100	38	99.6	76
4/ 7/21	15	5311	4.4	8.4	12.8	13.5	101	38	99.4	72
4/ 9/21	17	5319	3.9	9.2	13.1	13.8	101	36	99.9	80
4/11/21	19	5326	4.1	9.1	13.2	14.0	108	41	100.0	86
4/13/21	21	5336	4.2	8.4	12.6	13.5	101	39	100.1	74
4/15/21	23	5352	4.5	8.0	12.5	14.1	101	42	100.0	88
4/18/21	26	5364	4.2	8.9	13.1	14.2	104	38	99.6	84
4/20/21	28	5381	4.3	8.5	12.8	14.0	101	37	100.0	90
4/22/21	30	5390	4.3	8.7	13.0	14.1	104	36	100.0	90
4/25/21	33	5398	4.7	8.2	12.9	14.3	106	41	100.1	92
4/27/21	35	5405	4.2	8.3	12.5	13.9	101	39	99.8	88

* Case 10, J. S., a man, aged 28 years, had pneumonia of the left lower lobe. Pneumococcus, Group IV, was present. The patient recovered.

TABLE 16.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data	
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Maximum Temperature	Maximum Pulse
2/14/21	6	....	...	...	....	....	...	..	104.2	116
2/15/21	7	5037	3.5	8.8	12.3	13.1	79	27	101.0	100
2/17/21	9	5051	3.8	8.8	12.6	13.3	82	27	98.8	84
2/18/21	10	5059	4.2	8.4	12.6	13.2	81	26	98.8	84
2/21/21	13	5072	4.0	8.5	12.5	13.0	81	25	99.6	80
2/22/21	14	.....	...	...	....	....	...	..	99.0	96
2/23/21	15	5080	3.7	8.7	12.4	12.7	77	24	99.6	84
2/25/21	17	5087	3.9	8.2	12.1	12.5	76	26	99.6	96
2/28/21	20	5101	3.3	8.8	12.1	12.4	77	25	99.4	100

* Case 11, S. B., a youth, aged 13 years, had pneumonia of the right upper lobe. Pneumococcus, Type I, and B. influenzae were present. The patient recovered.

TABLE 17.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of disease.	Roentgenographic Data							Clinical Data				
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Serum, C.c.	Serum, Disease	Digitan, Gm.	Maximum Temperature	Maximum Pulse
2/ 6/21	4	....	...	...	....	....	...	..	...	..	...	104.4	120
2/ 7/21	5	5004	5.1	9.0	14.1	15.4	120	40	200	..	0.2	106.7	156
2/ 8/21	6	....	...	...	....	....	...	..	100	..	0.4	103.4	128
2/11/21	9	5024	5.3	8.6	13.9	15.3	119	41	...	..	...	99.3	98
2/14/21	12	5034	5.2	8.4	13.6	15.0	110	39	...	+	...	99.2	92
2/16/21	14	5046	4.8	8.9	13.7	15.2	114	40	...	+	...	99.4	100
2/19/21	17	5062	4.4	9.6	14.0	15.6	114	38	...	+	...	99.3	82
2/21/21	19	5070	4.8	9.4	14.2	15.6	120	38	...	+	...	99.0	84
2/23/21	21	5082	5.6	8.1	13.7	14.6	109	38	...	+	...	99.8	94
2/25/21	24	5088	5.5	8.6	14.1	15.5	121	38	...	..	...	98.5	88
2/28/21	27	5099	6.1	7.6	13.7	15.1	110	37	...	..	...	99.3	78
3/ 3/21	29	5126	5.3	8.3	13.6	15.0	108	38	...	..	...	99.0	96
3/ 7/21	33	5144	5.3	8.2	13.5	15.0	113	35	...	..	...	99.6	80

* Case 12, J. C., a man, aged 30 years, had pneumonia of the right lower lobe. Pneumococcus, Type I, was present. The patient recovered.

TABLE 18.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of disease.	Roentgenographic Data: Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Clinical Data: Serum, C.c.	Serum, Disease	Digitan, Gm.	Maximum Temperature	Maximum Pulse
3/22/21	4	5225	4.1	11.7	15.8	17.4	138	35	180	..	0.4	104.4	106
3/23/21	5	....	...	....	....	....	...	..	90	..	0.4	102.6	92
3/24/21	6	5243	4.9	11.0	15.9	17.5	141	34	...	..	...	100.6	66
3/26/21	8	5257	5.1	10.3	15.4	17.5	139	39	...	..	...	99.7	80
3/28/21	10	5263	4.8	10.9	15.7	17.0	139	33	...	+	...	99.4	60
3/30/21	12	5272	4.4	10.2	14.6	15.9	119	32	...	+	...	101.6	92
4/ 1/21	14	5286	4.6	10.1	14.7	16.2	124	35	...	+	...	99.2	80
4/ 4/21	17	5298	4.9	10.1	15.0	16.5	127	33	...	..	...	99.4	76
4/ 6/21	19	5310	4.7	10.7	15.2	16.3	129	30	...	..	...	99.2	64
4/ 8/21	21	5317	4.5	10.4	14.9	16.0	122	30	...	..	...	99.1	84
4/11/21	24	5327	4.7	10.8	15.5	16.8	124	32	...	..	...	99.1	82
4/13/21	26	5337	4.2	11.8	16.0	16.7	128	28	...	..	...	99.0	84

* Case 13, P. O'M., a man, aged 25 years, had pneumonia of the right upper, middle and lower lobes. Pneumococcus, Type I, was present. The patient recovered.

TABLE 19.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of disease.	Roentgenographic Data: Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Clinical Data: Serum, C.c.	Serum, Disease	Digitan, Gm.	Maximum Temperature	Maximum Pulse
11/11/21	4	....	...	...	...	....	...	..	...	..	1.0	104.0	112
11/12/21	5	5798	5.5	8.3	13.8	15.0	110	39	200	..	0.5	103.6	96
11/13/21	6	....	...	...	....	....	...	..	200	..	...	102.0	100
11/14/21	7	5800	6.0	7.3	13.3	14.5	106	39	100	..	...	101.2	108
11/16/21	9	5810	6.1	7.9	14.0	14.8	112	36	...	..	...	101.2	82
11/18/21	11	5816	5.6	7.8	13.4	14.6	109	39	...	..	...	100.8	92
11/19/21	12	....	...	...	....	....	...	..	...	+	...	102.6	96
11/22/21	15	5824	5.8	7.2	13.0	14.4	103	39	...	+	...	102.4	104
11/23/21	16	5828	5.4	8.5	13.9	15.4	115	39	...	+	...	102.8	116
11/25/21	18	5833	5.4	8.7	14.1	15.5	116	38	...	+	...	103.5	120
11/28/21	21	5839	5.7	8.4	14.1	15.9	118	41	...	..	...	103.0	120
11/30/21	23	5853	5.1	8.9	14.0	15.9	117	39	...	..	...	101.8	102
12/13/21	36	5907	5.3	9.1	14.4	15.3	113	35	...	..	...	99.4	90
12/15/21	38	5913	5.9	8.8	14.7	15.8	114	35	...	..	...	99.1	82
12/19/21	42	5933	4.9	9.2	14.1	15.3	107	35	...	..	...	99.6	88
12/21/21	44	5938	5.1	8.4	13.5	15.2	103	37	...	..	...	100.0	84

* Case 14, E. D., a man, aged 39 years, had pneumonia of the left lower and right upper lobes. Pneumococcus, Type I, was present. The patient recovered.

TABLE 20.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data		
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Digitan, Gm.	Maximum Temperature	Maximum Pulse
11/14/19	3	....	...	...	....	....	...	..	...	104.2	118
11/15/19	4	3962	3.8	9.4	13.2	15.5	115	41	...	104.4	120
11/17/19	6	3966	4.0	9.6	13.6	17.0	136	48	1.0	103.9	127
11/19/19	8	....	...	...	....	....	...	..	0.5	103.0	138
11/21/19	10	3983	3.7	9.1	12.8	15.9	116	47	...	101.6	98
11/24/19	13	3994	4.0	7.7	11.7	15.1	107	50	...	101.4	82
11/26/19	15	4007	3.5	8.0	11.5	14.8	103	48	...	100.6	80
11/28/19	17	4013	3.2	8.7	11.9	15.4	109	46	...	100.2	90
12/ 1/19	20	4027	2.9	8.7	11.6	14.7	102	32	...	100.3	100
12/ 3/19	22	4041	3.0	8.7	11.7	15.0	104	45	...	100.2	86
12/ 5/19	24	4058	3.1	9.0	12.1	14.9	106	44	...	100.3	88
12/ 8/19	27	4067	3.5	8.1	11.6	14.9	102	46	...	100.2	88
12/10/19	29	4078	3.8	7.6	11.4	14.2	99	46	...	100.0	96
12/12/19	31	4089	3.2	8.2	11.4	14.1	101	45	...	99.0	88
12/15/19	34	4097	3.7	8.0	11.7	14.2	100	45	...	98.5	80
12/17/19	36	4107	3.6	7.8	11.4	14.6	101	49	...	98.0	84
12/20/19	39	4119	3.8	7.7	11.5	14.8	103	48	...	98.5	80
12/24/19	43	4131	4.0	7.2	11.2	14.8	104	50	...	98.5	88
2/ 9/21	Returned	5003	4.3	7.6	11.9	13.6	102	45	...	.....	..

* Case 15, A. T., a man, aged 29 years, had pneumonia of the right upper, middle and lower lobes and of the left lower lobe. Pneumococcus, Type III, was present. The patient recovered.

TABLE 21.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data		
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Digitan, Gm.	Maximum Temperature	Maximum Pulse
10/12/21	3	....	...	....	....	....	...	..	...	104.4	98
10/13/21	4	5681	5.3	9.8	15.1	15.0	108	27	1.0	102.6	96
10/14/21	5	....	...	....	....	....	...	..	0.5	103.2	96
10/15/21	6	5689	5.0	9.7	14.7	14.4	107	26	...	102.4	88
10/18/21	9	5695	5.6	10.1	15.7	15 1	115	24	...	100.2	76
10/20/21	11	5704	5.0	11.1	16.1	15.4	115	22	...	99.8	60
10/22/21	13	5714	5.5	10.7	16.2	15.8	119	23	...	99.6	68
10/24/21	15	5719	4.9	11.0	15.9	15.7	114	24	...	99.7	68
10/26/21	17	5728	4.5	11.3	15.8	15.5	114	21	...	99.6	74

* Case 16, F. L., a man, aged 32 years, had pneumonia of the left lower lobe and chronic heroin poisoning. Pneumococcus, Group IV, was present. The patient recovered from pneumonia but the second condition was unimproved.

TABLE 22.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data				
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq Cm.	Angle, Degrees	Serum, C.c.	Serum, Disease	Digitan, Gm.	Maximum Temperature	Maximum Pulse
2/23/21	4	....	...	...	....	....	...	..	...	..	...	104.6	120
2/24/21	5	5085	5.1	7.5	12.6	12.9	99	32	90	..	0.3	105.0	112
2/25/21	6	....	...	...	....	....	...	..	180	..	0.7	104.2	104
2/26/21	7	5097	5.4	7.2	12.6	13.0	100	34	...	..	0.5	100.6	98
3/ 1/21	10	5112	5.0	7.1	12.1	12.7	97	36	...	..	...	100.3	80
3/ 3/21	12	5127	5.0	6.7	11.7	12.3	94	38	...	..	...	99.9	92
3/ 4/21	13	....	...	...	....	....	...	..	...	+	...	100.4	92
3/ 5/21	14	5137	5.5	6.5	12.0	12.5	95	37	...	+	...	101.9	90
3/ 7/21	16	5143	5.4	6.6	12.0	12.2	96	34	...	..	...	100.6	100
3/ 9/21	18	5152	5.5	6.3	11.8	12.4	95	37	...	..	...	100.6	88
3/11/21	20	5159	5.5	6.5	12.0	12.6	99	38	...	..	...	99.3	88
3/14/21	23	5170	5.2	7.0	12.2	12.9	100	35	...	..	...	100.0	84
3/16/21	25	5186	5.4	6.6	12.0	12.3	96	37	...	..	...	100.0	92
3/18/21	27	5195	5.4	6.9	12.3	12.7	96	34	...	..	...	99.2	90
3/21/21	30	5212	5.8	6.2	12.0	12.8	100	42	...	..	...	99.6	88
3/23/21	32	5231	5.2	6.7	11.9	13.0	101	39	...	..	...	100.0	90

* Case 17, V. P., a man, aged 26 years, had pneumonia of the right lower lobe and otitis media of the left ear. Pneumococcus, Type I, was recovered from the sputum; hemolytic streptococci and staphylococci were present in the discharge from the ear. The patient recovered.

TABLE 23.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data				
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Serum, C.c.	Serum, Disease	Digitan, Gm.	Maximum Temperature	Maximum Pulse
1/ 4/22	2	....	...	...	....	....	...	..	...	...	0.8	104.0	120
1/ 5/22	3	5985	5.6	7.7	13.3	13.9	100	39	100	...	...	105.3	128
1/ 6/22	4	6006	5.5	8.0	13.5	14.2	100	44	...	...	0.1	101.4	118
1/ 9/22	7	6013	5.0	7.6	12.6	14.0	106	42	...	...	...	99.6	92
1/11/22	9	6035	4.7	7.6	12.3	13.8	98	40	...	+†	...	99.4	80
1/14/22	12	6042	4.2	8.4	12.6	13.7	101	37	...	...	...	99.1	88
1/16/22	14	6052	3.6	9.1	12.7	14.1	103	34	...	...	...	99.8	78
1/18/22	16	6068	4.0	8.0	12.0	13.9	99	38	...	...	...	99.1	92
1/20/22	18	6082	4.2	8.5	12.7	13.8	100	38	...	...	...	99.4	80
1/23/22	21	6093	3.6	9.3	12.9	13.9	101	37	...	...	...	99.4	80
1/25/22	23	6109	4.9	8.4	13.3	14.0	104	35	...	...	...	99.2	88

* Case 18, M. L., a woman, aged 34 years, had pneumonia of the left lower lobe. Pneumococcus, Type I, and B. influenzae were present. The patient recovered.

† Serum disease began on the ninth day and continued through the eleventh day.

TABLE 24.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data: Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Clinical Data: Digitan, Gm.	Maximum Temperature	Maximum Pulse
10/21/21	2	....	...	...	....	....	...	..	0.4	105.2	138
10/22/21	3	5716	4.6	8.6	13.2	13.9	108	35	1.0	104.0	136
10/23/21	4	....	...	...	....	....	...	..	0.4	102.8	116
10/24/21	5	5718	4.3	9.3	13.6	14.2	114	32	...	101.1	100
10/26/21	7	5727	4.6	8.9	13.5	13.9	113	29	...	99.1	80
10/28/21	9	5744	5.3	8.3	13.6	13.9	112	30	...	98.8	76
10/31/21	12	5755	4.9	8.2	13.1	13.3	107	36	...	99.4	78
11/ 2/21	14	5761	4.6	8.7	13.3	13.7	111	35	...	99.5	92
11/ 4/21	16	5771	4.7	8.4	13.1	13.4	109	34	...	99.0	84

* Case 19, L. W., a youth, aged 16 years, had pneumonia of the right lower lobe. Pneumococcus, Type II, and B. influenzae were present. The patient recovered.

TABLE 25.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data: Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Clinical Data: Digitan, Gm.	Maximum Temperature	Maximum Pulse
12/27/21	5	5961	4.5	9.1	13.6	14.4	111	33	...	104.5	106
12/28/21	6	....	...	...	....	....	...	..	0.4	104.2	98
12/29/21	7	....	...	...	....	....	...	..	0.5	103.4	84
12/30/21	8	5965	4.9	9.2	14.1	14.3	108	30	...	99.6	72
1/ 3/22	12	5970	5.2	8.8	14.0	14.9	112	34	...	98.6	66
1/ 5/22	14	5986	5.2	8.6	13.9	14.5	108	34	...	99.0	72
1/ 7/22	16	6004	5.1	9.7	14.8	15.2	114	28	...	98.9	64
1/ 9/22	18	6010	4.8	9.1	13.9	14.4	104	32	...	99.0	82
1/11/22	20	6033	4.6	9.8	14.4	14.7	111	29	...	99.4	72
1/14/22	23	6038	5.0	9.3	14.3	14.8	110	31	...	99.4	74

* Case 20, A. R., a man, aged 37 years, had pneumonia of the right upper lobe. Pneumococcus, Group IV, was present. The patient recovered.

TABLE 26.

*Findings in a Case of Lobar Pneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data		
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Digitan, Gm.	Maximum Temperature	Maximum Pulse
1/ 1/22	2	....	...	...	....	....	...	..	0.6	102.6	116
1/ 2/22	3	....	...	...	....	....	...	..	0.4	100.0	69
1/ 3/22	4	5971	4.0	10.0	14.0	14.8	114	42	...	99.5	65
1/ 5/22	6	5987	3.9	10.4	14.3	14.9	118	36	...	99.4	65
1/ 6/22	7	6005	4.1	10.3	14.4	14.7	116	31	...	99.4	65
1/ 9/22	10	6011	4.3	9.9	14.2	14.8	113	35	...	99.0	68
1/11/22	12	6034	4.2	10.3	14.5	15.0	120	33	...	99.2	76
1/16/22	17	6049	4.1	10.3	14.4	14.7	115	36	...	98.4	65
1/18/22	19	6064	4.0	10.1	14.1	14.6	116	33	...	98.4	70

* Case 21, R. L., a man, aged 34 years, had pneumonia of the left lower lobe. Pneumococcus, Group IV, and B. influenzae were present. The patient recovered.

TABLE 27.

*Findings in a Case of Bronchopneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data	
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Maximum Temperature	Maximum Pulse
12/ 3/19	8	.....	...	....	....	....	...	..	103.4	100
12/ 4/19	9	4048	5.4	11.4	16.8	18.0	142	28	102.6	102
12/ 6/19	11	4063	5.4	11.4	16.8	17.9	146	26	101.6	94
12/ 8/19	13	4072	5.3	12.1	17.4	18.4	150	26	101.0	84
12/12/19	17	4093	5.1	11.7	16.8	17.9	143	26	101.0	88
12/15/19	20	4102	4.3	11.9	16.2	17.0	128	24	100.5	88
12/17/19	22	4111	5.5	11.7	17.2	17.7	139	18	100.6	80
12/19/19	24	4117	4.0	13.0	17.0	17.8	138	21	100.7	80
12/22/19	27	4124	4.0	13.3	17.3	18.3	139	21	100.7	80
12/24/19	29	4136	4.3	12.4	16.7	17.3	134	20	98.8	78
12/30/19	35	4142	4.4	12.8	17.2	17.8	137	19	98.6	88
1/ 2/20	38	4150	4.8	12.7	17.5	18.1	135	21	97.8	76
1/ 5/20	41	4157	4.4	12.0	16.4	17.2	137	22	98.6	84
2/ 2/21	Returned	4966	5.7	12.2	17.9	18.7	146	23	....	...

* Case 22, M. Q., a man, aged 39 years, had diffuse bilateral bronchopneumonia. Pneumococcus, Group IV, and B. influenzae were present. The patient recovered.

TABLE 28.

*Findings in a Case of Bronchopneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data	
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Maximum Temperature	Maximum Pulse
11/14/19	3	....	...	....	....	....	...	..	103.0	116
11/15/19	4	3959	3.7	10.2	13.9	14.8	103	39	105.4	112
11/17/19	6	3665	4.0	10.5	14.5	16.4	121	44	104.1	110
11/19/19	8	3976	4.6	10.3	14.9	17.3	135	42	101.0	98
11/21/19	10	3980	4.1	10.6	14.7	16.5	128	41	102.0	96
11/24/19	13	3993	4.1	10.5	14.6	16.0	122	39	100.3	92
11/26/19	15	4005	3.8	10.6	14.4	15.2	124	37	100.5	88
11/28/19	17	4018	3.6	10.7	14.3	15.2	118	37	100.4	88
12/ 1/19	20	4025	3.4	11.0	14.4	15.2	115	35	100.0	84
12/ 3/19	22	4039	4.0	10.1	14.1	14.5	108	38	100.6	96
12/ 5/19	24	4057	4.1	9.2	13.3	14.4	104	37	100.2	100
12/ 8/19	27	4065	4.3	8.8	13.1	14.0	114	43	99.0	98
12/10/19	29	4079	3.5	9.9	13.4	14.2	109	37	98.7	100
12/12/19	31	4088	4.0	10.4	14.4	14.5	111	35	98.7	108

* Case 23, E. S., a woman, aged 55 years, had bronchopneumonia of the left lower lobe. Pneumococcus, Group IV, was present. The patient recovered.

TABLE 29.

*Findings in a Case of Bronchopneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data	
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Maximum Temperature	Maximum Pulse
11/18/19	2	....	...	...	....	....	...	..	104.0	126
11/19/19	3	3972	4.0	8.2	12.2	14.2	106	50	102.4	110
11/21/19	5	3982	4.1	8.0	12.1	13.6	104	47	100.0	84
11/24/19	8	3991	3.9	8.2	12.1	13.5	102	47	100.0	80
11/26/19	10	4003	3.8	8.5	12.3	14.3	102	49	99.4	80
11/27/19	11	....	...	...	....	....	...	..	100.0	78
11/28/19	12	4016	3.8	8.3	12.1	13.7	104	45	99.6	80
12/ 1/19	15	4026	4.0	8.3	12.3	13.5	103	46	98.6	80
12/ 3/19	17	4037	3.6	8.6	12.2	13.7	100	46	99.3	88
12/ 5/19	19	4056	3.7	8.4	12.1	13.6	98	45	98.6	80
12/ 8/19	22	4066	3.4	8.6	12.0	13.6	100	42	98.8	80

Case 24, A. J., a woman, aged 27 years, had bronchopneumonia of the right lower lobe. Pneumococcus, Group IV, was present. The patient recovered.

TABLE 30.

*Findings in a Case of Bronchopneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data	
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Maximum Temperature	Maximum Pulse
2/ 3/21	5	4976	4.0	8.5	12.5	13.0	101	33	101.0	90
2/ 9/21	11	5005	4.0	8.4	12.4	12.8	97	35	99.2	70
2/11/21	13	5021	4.1	8.1	12.2	12.7	97	30	99.6	94
2/14/21	16	5033	4.0	8.3	12.3	12.8	98	32	98.3	70

* Case 25, G. B., a girl, aged 13 years, had bronchopneumonia of the right lower lobe. Streptococcus hemolyticus was present. The patient recovered.

TABLE 31.

*Findings in a Case of Bronchopneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data	
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Maximum Temperature	Maximum Pulse
1/ 9/20	7	....	...	...	....	....	...	..	103.2	100
1/10/20	8	4173	5.2	9.0	14.2	14.5	118	30	102.8	92
1/12/20	10	4177	5.6	8.5	14.1	14.7	113	31	103.5	116
1/14/20	12	4186	5.0	9.2	14.2	14.5	113	30	103.0	120
1/16/20	14	4194	4.9	9.2	14.1	14.5	112	29	102.8	116
1/19/20	17	4205	5.6	7.3	12.9	13.6	105	36	105.8	128

* Case 26, C. G., a man, aged 35 years, had diffuse bilateral bronchopneumonia. Hemolytic streptococcus and B. influenzae were present. The patient died.

TABLE 32.

*Findings in a Case of Bronchopneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data		
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Digitan, Gm.	Maximum Temperature	Maximum Pulse
11/23/21	5	....	...	...	....	....	...	..	...	105.3	110
11/24/21	6	....	...	...	....	....	...	..	0.6	104.9	108
11/25/21	7	5834	4.6	8.3	12.9	14.5	107	43	0.4	105.2	114
11/29/21	11	5847	4.6	8.1	12.7	14.2	106	42	...	99.0	108
12/ 1/21	13	5865	4.3	9.1	13.4	15.0	109	40	...	100.4	88
12/ 5/21	17	5876	4.7	8.1	12.8	14.9	106	44	...	98.5	80
12/ 7/21	19	5880	4.5	8.4	12.9	14.9	106	42	...	99.4	86
12/ 9/21	21	5891	4.6	8.6	13.2	14.8	109	41	...	99.4	86
12/12/21	24	5899	4.0	8.8	12.8	14.8	105	43	...	99.6	88
12/14/21	26	5909	4.6	8.8	13.4	15.1	109	41	...	99.8	88
12/19/21	31	5932	4.2	8.6	12.8	14.5	104	42	...	99.2	90
12/21/21	33	5936	4.1	9.1	13.2	14.9	107	40	...	99.5	94

* Case 27, M. K., a youth, aged 19 years, had bronchopneumonia of the right lower lobe. Pneumococcus, Group IV, was present. The patient recovered.

TABLE 33.

Findings in a Case of Bronchopneumonia.

Date	Day of Disease	Roentgenographic Data							Clinical Data		
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Strophanthin, Gm.	Maximum Temperature	Maximum Pulse
1/ 6/20	7	4161	4.4	7.2	11.6	12.9	89	37	...	104.0	84
1/ 7/20	8	4166	3.7	7.6	11.3	12.9	89	38	0.7	104.2	86
1/ 9/20	10	4172	4.2	7.2	11.4	12.5	85	36	...	100.5	70
1/12/20	13	4175	4.4	7.3	11.7	13.1	91	36	...	99.3	66
1/14/20	15	4183	4.3	7.6	11.9	13.0	93	35	...	98.6	70
1/16/20	17	4191	4.5	7.2	11.7	13.0	90	38	...	98.6	66
1/19/20	20	4199	4.3	7.2	11.5	12.7	87	35	...	98.8	72
1/21/20	22	4213	4.1	7.4	11.5	12.8	89	36	...	98.8	79
1/23/20	23	4226	4.5	7.0	11.5	12.8	89	36	...	99.3	88

* Case 28, A. D. P., a woman, aged 34 years, had bronchopneumonia of the right lower lobe, chronic endocarditis and mitral stenosis. Staphylococcus aureus and albus and B. influenzae were present. The patient recovered.

TABLE 34.

*Findings in a Case of Bronchopneumonia.**

Date	Day of Disease	Roentgenographic Data							Clinical Data		
		Plate No.	Maximum Right, Cm.	Maximum Left, Cm.	Transverse Diameter, Cm.	Long Diameter, Cm.	Area, Sq. Cm.	Angle, Degrees	Strophanthin, Gm.	Maximum Temperature	Maximum Pulse
11/23/19	7	....	...	...	....	....	..	..	...	102.8	124
11/24/19	8	3995	4.1	5.9	10.1	10.9	81	50	...	104.6	140
11/26/19	10	4006	4.1	5.7	9.8	10.7	77	51	...	103.3	135
11/28/19	12	4017	4.0	6.1	10.1	10.7	79	49	...	102.8	136
12/ 7/19	21	....	...	...	....	....	..	..	0.2	102.8	170
12/10/19	24	....	...	...	....	....	..	..	0.3	104.4	176

* Case 29, G. E., a boy, aged 7 years, had diffuse bilateral bronchopneumonia. Pneumococcus, Group IV, and B. influenzae were present. The patient died.

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THE FORMOL TITRATION OF BACTERIOLOGICAL MEDIA.

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PLATES 1 TO 3.

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The principles involved in the "formol titration" of bacterial cultures of media are the same as those embodied in the methods of Malfatti (1908) and of Henriques and Sörensen (1909) for the titration of urine. They have been applied to the study of bacterial cultures by a number of authors, notably Kendall, Day and Walker (1913), Itano (1916), Berman and Rettger (1918), Foster and Randall (1921), Ayers, Rupp and Mudge (1921), and Kendall (1922). An excess of formalin (approximately 40 per cent formaldehyde) is added to the sample to be titrated. The sample becomes more acid as a result of the following type reactions:

$$\begin{array}{l} R \cdot CH \cdot NH_2 \\ \quad | \\ \quad COOH \end{array} + CH_2O = \begin{array}{l} R \cdot CH \cdot N : CH_2 \\ \quad | \\ \quad COOH \end{array} + H_2O$$

$$4NH_4\,Cl + 6CH_2O = N_4(CH_2)_6 + 4HCl + 6H_2O$$

The increase in acidity is then titrated with a standard alkali solution. Ammonia, primary amines, and the amino groups of amino-acids, and polypeptids react with formaldehyde. The titration, therefore, represents the sum of these substances.

Sörensen (1907) (1908) has pointed out that the reaction of amino-acids with formaldehyde is a reversible reaction, that an excess of formaldehyde tends to throw the reaction, as expressed in the above equation, from left to right, whereas increasing amounts of water throw the reaction from right to left. Since the methylene derivative of the amino-acid is a stronger acid than the amino-acid and is therefore more easily titrated, it is obviously desirable to force

the reaction from left to right. To do this Sörensen found it necessary to add as much as 10 cc. of formalin to 20 cc. of sample (amino-acid solution), and to titrate with N/5 barium hydroxide or sodium hydroxide. Smaller amounts of formalin were insufficient; larger amounts unnecessary. Workers in bacteriology appear not to have appreciated the importance of this factor. Working with solutions of pure amino-acids Sörensen found it necessary to carry the titration to an end point on the alkaline side of pH 8.2 in order to approximate the theoretical value of some of the amino-acids. He therefore titrated to a deep red color with phenolphthalein as an indicator which he states to have been at about pH 9.0, or to a strong blue color with thymolphthalein which he states to have been at about pH 9.7. Sörensen recognized the presence of carbonates and phosphates as a serious source of error in the formol titrations of amino-acid mixtures containing these substances and to minimize this difficulty titrated with barium hydroxide rather than sodium hydroxide, also adding barium chloride to depress the ionization of the barium carbonate and phosphate formed. The methylene derivatives of some polypeptids and certain of the amino-acids, however, notably tyrosine and phenylalanine, formed insoluble precipitates with barium and for their titration better results were obtained with sodium hydroxide. In the methods of Henriques (1909) and of Henriques and Sörensen (1909) the barium carbonate and phosphate were filtered out, the filtrate neutralized, formalin added, and the acid titrated against sodium or potassium hydroxide.

In developing his method Sörensen made formol titrations of pure solutions of many of the amino-acids and of some peptic, tryptic, and ereptic digests of Witte peptone, casein, and egg albumin. Henriques and Sörensen titrated urines. For the titration of bacterial cultures Itano (1916) followed quite closely the method of Sörensen. Kendall and his associates departed widely from the method of Sörensen in that they used a much larger proportion of water (50 cc.) and a smaller proportion of formalin (5 cc.). Foster and Randall (1921) followed the technic of Kendall, Day and Walker. Berman and Rettger (1918) departed even more widely from the technic of Sörensen in that they used a total volume of 55 cc. of water and only 2 cc. of formalin. Ayers, Rupp and Mudge (1921) presumably

followed Sörensen since they do not mention any modification of the technic. Kendall, Day and Walker and Berman and Rettger departed from Sörensen's method in another respect. They "neutralized" their samples of media to the end point of phenolphthalein, a point which Kendall defines as pH 8.3, before adding the formalin. Sörensen (1908) did not do this. He says, after describing the preparation of the control solution to the "deutliche rote Farbe (zweites Stadium)" of phenolphthalein: "Die zur Untersuchung vorliegenden Lösungen werden bis zu dieser letzten Farbenstärke titriert, indem 20 ccm der Analyse 10 ccm Formolmischung zugesetzt werden, und gleich darauf n/5 Barytlauge bis Rotfärbung, ... "

In the French edition (1907) of his article the meaning is equally clear. He says: "Ensuite on titre jusqu'à la même intensité de coloration les liqueurs à examiner; à cet effet, on ajoute à 20 cc. de la liqueur 10 cc. du mélange de formol et immédiatement après, en agitant bien, la solution de baryte au 1/5, jusqu'à ce que le liquide devienne rouge, ... "

It seems worth while quoting the above passages verbatim because the description of Sörensen's method in certain text books of physiological chemistry would lead one to believe that the solution to be analyzed should be titrated to the color of phenolphthalein produced in the second stage of the control before the formalin is added.

For the titration of solutions of pure amino-acids Sörensen (1907) (1908) apparently carried out no preliminary neutralization or adjustment of hydrogen ion concentration, though when it was necessary to add alkali to get the substance into solution (e.g., tyrosine and aspartic acid) the amount of alkali added was considered in the computation of the formol titration. Later Henriques and Sörensen (1909) (1910) pointed out that the neutral point of the amino-acids is at about pH 6.8 and that this therefore should be the starting point for the titration. They brought their samples to this reaction by means of a specially prepared litmus paper. Northrop (1921) adopted pH 7.0 with neutral red as an indicator for the starting point of the titration of hydrolyzed gelatin. H. Jessen-Hansen in Abderhalden's Handbuch der biochemischen Arbeitsmethoden, vi, 262, gives a very good discussion of the importance of this factor. Theoretically the isoelectric point should be the point of departure for

the titration of amino-acids. Fortunately for our purpose the isoelectric points of the various amino-acids are so close together that their isoelectric zones (Michaelis, 1914, p. 40) overlap and pH 6.8 or 7.0 may be chosen as the ideal starting point for the titration of mixtures of amino-acids and ammonium salts.

The experiments and curves which follow serve to illustrate the importance of choosing the correct hydrogen ion concentrations for the initial and end points of the formol titrations. In bacteriological culture fluids we encounter not only mixtures of amino-acids and ammonium salts but also peptones, polypeptids, fatty acids, carbonates, phosphates, and carbohydrates. The reaction may be acid or alkaline. There are also the disturbing factors of color and turbidity. By known methods of precipitation, filtration, etc., it is possible to eliminate the disturbing factors in such a mixture and to make an accurate formol titration. The present paper is an attempt to analyze the factors involved and to reduce the formol titration of bacteriological media and cultures to its simplest terms.

Phenol red or thymol blue have been used as indicators. The end point is judged by comparison with colorimetric hydrogen ion standards in a comparator block. This not only leads to much greater accuracy in judging the end point but makes it possible to use a color screen of the material being titrated, doing away with the necessity for using artificial colors in a control as was done by Sörensen. Since this work has been in progress Northrop (1921) has also made similar use of the comparator block and colorimetric standards. The titration is carried on in large test tubes containing 1 cc. of sample and 9 cc. of distilled water. The alkali used is N/20 NaOH. For none of the media or cultures titrated to date has it been necessary to use more than 3 cc. of the alkali solution.

As a result of numerous experimental titrations conducted at various hydrogen ion concentrations it is found that optimum results are obtained when 8 cc. of formalin are added to the 10 cc. of diluted sample. Lesser amounts of formalin give lower titrations. It is desirable to use no more formalin than necessary because of the high buffer effect of the formalin itself which results in a poor end point if the titration is carried beyond pH 8.0. More than 8 cc. of formalin have been found unnecessary. The effect of using different amounts of formalin is illustrated in Table I.

In the titrations hereafter reported it is to be understood that 8 cc. of formalin were added to 10 cc. of the diluted sample.

There remain to be determined the limits of hydrogen ion concentration between which titration shall be conducted. If one were to titrate an amino-acid directly against sodium hydroxide, i.e., without the use of formalin, he should start the titration at the isoelectric point of the amino-acid and end it at the point of complete neutralization of the amino-acid. In the case of glycine this would be from pH 6.1 to about pH 11.3. However in the case of glycine and other amino-acids the isoelectric point is in a broad isoelectric zone extending in the case of glycine from about pH 5.0 to pH 7.5

TABLE I.

Effect of Various Amounts of Formalin on the Formol Titration.

Sample (1 cc. + 9 cc. H_2O).	Amount of formalin.			
	1 cc.	5 cc.	8 cc.	10 cc.
Alanine	1.16*	1.94	2.0	2.0
Bouillon	0.9	1.03	1.09	1.09

* The figures represent cc. of N/20 NaOH required for the titration of 1 cc. of sample, the formalin blank[2] having been determined for each amount of formalin and subtracted.

or 8.0. Within this zone the degree of dissociation (a) is very slight and even at pH 8.0 is less than 2 per cent.

$$a = \frac{k_a}{k_a + (H^+)} = \frac{1.8 \times 10^{-10}}{1.8 \times 10^{-10} + 1 \times 10^{-8}} = \frac{1.8}{101.8} \text{ at pH } 8.0$$

If a pure monocarboxylic amino-acid is dissolved in distilled water the solution will be found to have a hydrogen ion concentration at or near the isoelectric point, a fact also noted by Eckweiler, Noyes and Falk (1921). If it is titrated with alkali and the titration curve plotted with amounts of alkali added as abscissae and hydrogen ion exponents as ordinates, as was done in the curves to the left in Plate 1, the curve will be seen to drop almost vertically towards the alkaline side as the first drops of alkali are added. This represents a portion of the isoelectric zone. Asparagine (Plate 2) behaves as

a mono-carboxylic amino-acid since in this amide one of the carboxylic groups of aspartic acid is in combination with an amide group. Since by the formol titration it is the carboxyl radical and not the amino radical which is titrated it is important that the mono-amino-dicarboxylic acids be converted into mono-carboxylic acids, otherwise their titer will be doubled. This may be accomplished by titrating them with sodium hydroxide to the isoelectric point (or to within the isoelectric zone) of their mono-sodium salts. This zone is represented by the nearly vertical portion of the curves in Plate 2. To titrate the nitrogen equivalent of the mono-amino-dicarboxylic acids it is therefore necessary to start the titration within the isoelectric zone of their mono-sodium salts and this zone lies within the same region as that of the mono-carboxylic amino-acids.

Within the isoelectric zone a very minute amount of acid or alkali produces a marked change in hydrogen ion concentration. It makes scarcely any difference in titration at what point we start provided it is within the isoelectric zone. The sodium salts of the amino-acids are not titratable after the addition of formalin since they react with formaldehyde to produce not acids, but sodium salts of the methylene derivatives of amino-acids. In a solution of glycine at pH 8.4 $\left(a = \frac{1.8}{41.8}\right)$ about 4.3 per cent of the glycine is present as the sodium salt. If formalin is added at this point the reaction becomes more acid, or rather less basic, because of the formation of methylene derivatives. However there has already been added sufficient alkali to neutralize 4.3 per cent of the methylenglycine and unless this is taken into account, as was done by Sörensen when he dissolved amino-acids in alkali, the formol titration will fall short by a corresponding amount. Before the formalin is added, mixtures of amino-acids, protein digests, bacterial cultures, etc., should be brought to a reaction within the isoelectric zones of all the amino-acids present if this is possible or unless other considerations make it necessary to compromise in regard to this point. We will call this the initial point of the titration. The end point of the titration should be within what may be called the "zone of neutralization" of the amino-acid if it is titrated directly or within the zone of neutralization of the methylene derivative of the amino-acid if for-

malin has been added. The term "zone of neutralization" will be used to indicate that portion of the titration curve of an acid and base which extends, on either side of the neutral point, nearly vertically to the abscissa with increasing or decreasing hydrogen ion concentrations plotted as ordinates. The neutral point is the point of inflection of the curve within the zone of neutralization and is reached when just sufficient alkali has been added to convert all of the acid into its alkali salt. The neutral point in the titration of the methylene derivative of an amino-acid is at a higher hydrogen ion concentration than that in the titration of the amino-acid itself and it is upon this fact that the usefulness of the formol titration depends. If formalin is added to solutions of various amino-acids and these mixtures are then titrated with sodium hydroxide, the curves plotted on the right in Plates 1 and 2 are obtained. In these curves an almost vertical portion is harder to recognize. This is because of the great excess of formalin in the mixtures titrated. Of the 8 cc. of formalin added to each sample a very small fraction of 1 cc. actually reacts with the amino-acid to form the methylene derivative. The remainder exerts a powerful buffer effect on the alkaline side of pH 8.0. Commercial formalin is acid in reaction. It has been our practice to add to it in preparation for each day's work sufficient normal NaOH to reduce the acidity to about pH 5.0, then to titrate 8 cc. of it plus 10 cc. of distilled water to pH 9.0 or beyond with N/20 NaOH, to plot the titration curve, and use it for the day's experiments. In Plates 1, 2, and 3, points determined on these curves are indicated by crosses. If titration values on the formalin curve are subtracted from values at the same hydrogen ion concentration on the amino-acid plus formalin curve the differences may be plotted as a resultant curve lying between the other two. This resultant curve should be the titration curve of the methylene derivative of the amino-acid. Each one of these resultant curves does reach a nearly vertical position which is the zone of neutralization of the methylene derivative and is the end point of the titration. For each of the substances titrated this zone may be said to begin on the acid side at about the hydrogen ion concentration here listed.

	pH
Glycine	6.8
Phenylalanine	7.6 or 8.0
Asparagine	6.0
Glutamic acid	8.0
Alanine	8.0
Tyrosine	7.6 or 8.0
Aspartic acid	8.0

A similar zone is found in the resultant curve of the formol titration of ammonium salts (Plate 3). In these cases the resultant curve represents the titration of hydrochloric, lactic, phosphoric, or carbonic acid. The zones may be said to begin at the following hydrogen ion concentrations.

	pH
Ammonium chloride	6.0
Ammonium phosphate	8.0
Ammonium lactate	6.0
Ammonium carbonate	7.0

It will be noted that before the resultant curves reach pH 9.0 their course becomes somewhat irregular or uncertain. This is probably because in the titration of formalin and of formalin plus amino-acids the buffer effect of both substances is so great after they pass beyond pH 8.5 that the end point judged colorimetrically is a very broad and indefinite one. The effect of this is indicated in the cases of glycine, alanine, and asparagine (Plates 1 and 2) by shaded regions. The end points of the titrations might have been taken to lie at any point within these regions. The experimental or technical error is therefore quite large if the titration is carried much beyond pH 8.0 and is in our opinion too large for formol titration by the colorimetric method if carried beyond pH 9.0. The indicators used by us in these regions were phenol red and thymol blue. We found the end point with phenol red at pH 8.0 a very sharp one.[1] At pH 8.0 all of the substances titrated after the addition of formalin had reached the zone of neutralization. This then might be taken as the maximum hydrogen ion concentration at which the formol titration might

[1] Through the kindness of Dr. Van Slyke and Dr. Hastings of the Hospital of The Rockefeller Institute for Medical Research in New York City our color standard at this hydrogen ion concentration was checked electrometrically and found to be correct.

be completed. An end point of pH 8.2 or 8.4 might be a little better were it not for certain other factors peculiar to the titration of bacteriological media which are discussed below. On the other hand the curves show that pH 8.0 is a little too alkaline to be within the isoelectric zones of some of the amino-acids and ammonium salts. The ideal starting point for formol titration of mixtures of amino-acids would therefore be at a higher hydrogen ion concentration. A hydrogen ion concentration of pH 6.8 as recommended by Henriques and Sörensen (1909) (1910) or of pH 7.0 as employed by Northrop (1921) would serve very well.

However our problem is a special one in that bacteriological media and especially bacterial cultures in such media are more than mere mixtures of amino-acids. They contain among other things volatile and non-volatile fatty acids, phosphates and carbonates, and these substances, especially fatty acids and carbonates together with amino-acids and ammonium salts may change in amount during the growth of the culture. What influence do these substances exert within the range of hydrogen ion concentration chosen for the formol titration? At pH 7.0 the volatile fatty acids are practically neutralized so that they do not enter into a titration with sodium hydrate from pH 7.0 to pH 8.0. The phosphates and carbonates exert large buffer effects between pH 7.0 and pH 8.0, and the amount of alkali with which they are capable of combining will appear as an error in the formol titration between these limits. If therefore the solution to be titrated is brought to a certain hydrogen ion concentration (e.g., pH 7.0) formalin added and the mixture titrated to a lower hydrogen ion concentration (e.g., pH 8.0 or 8.4) it is absolutely necessary to get rid of the phosphates and carbonates. This may be done by precipitation with barium and filtration.

The following method has given the most accurate results with bacteriological media and with mixtures of amino-acids, phosphates and carbonates.

Method A.—Measure out accurately with an Ostwald pipette 2 cc. of culture medium. Add from a burette exactly 2 cc. of N/5 or stronger NaOH. Add a small piece (about 0.2 gram) of barium chloride. Shake to dissolve the barium chloride and allow to stand for a few minutes. Pour onto a small dry paper filter.

With the Ostwald pipette measure out 2 cc. of the filtrate into a large test tube (1 inch in diameter) such as can be used in the comparator block. Caution: Do not blow through the pipette used for measuring the sample since the CO_2 from the breath will cause the fluid to become cloudy.

Add 5 drops of phenol red solution and sufficient N/5 HCl to bring the reaction of the sample near pH 7.0 and then sufficient distilled water to bring the contents of the tube to about 10 cc. which may be judged by a mark on the side of the tube. Bring the reaction of the sample to pH 7.0 with N/20 HCl or NaOH, using the comparator block with 1 cc. of medium plus 9 cc. of water as a color screen behind the colorimetric standard.

Add 4 drops more of the phenol red solution and 8 cc. of formalin.[2] Titrate with N/20 NaOH to pH 8.0 or 8.4. From this result subtract the titration of the formalin blank.[2] The remainder multiplied by 5 is the formol titration expressed in terms of per cent normal, *i.e.*, the percentage normality of substances reacting with formaldehyde to produce titratable acids.

In the formol titration of bouillon we have obtained exactly the same values when titrating to pH 8.0 as at pH 8.4 whereas the colorimetric end point at pH 8.0 is somewhat sharper than at pH 8.4 because of the greater buffer effect of the formalin at pH 8.4.

A briefer method than the above, one which requires less material and which gives almost identical results even in the presence of large amounts of carbonates and phosphates in bouillon, is as follows:

Method B.—With an Ostwald pipette measure out 1 cc. of the medium or culture into each of two large test-tubes (1 inch in diameter) such as can be used in the comparator block. To each tube add 9 cc. of ammonia-free distilled water or better sufficient water to bring the contents of both tubes to the same level. One of the tubes serves as a color screen in the comparator block. To the other tube, hereafter referred to as the sample, add 5 drops of phenol red (the same proportion of indicator as is contained in the colorimetric hydrogen ion standards).

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[2] It is our experience that if formalin is made alkaline it does not "keep" well, *i.e.*, the hydrogen ion concentration increases slowly but appreciably within two hours. If, however, it is made slightly acid it does not change appreciably during the working day. Instead of adjusting the reaction of the formalin to pH 8.0 or 8.4 before adding it to the sample of medium we prefer to prepare it as follows: To a sufficient quantity for the day's work is added normal NaOH (usually less than 1 cc. per 100 cc. of formalin) until the reaction is between pH 5.0 and pH 7.0. The formalin blank consists of 8 cc. of this formalin plus 10 cc. of distilled water titrated against N/20 NaOH to pH 8.0 or 8.4 (the end point chosen for the formol titrations).

Bring the reaction of the sample to pH 8.0 by the cautious addition of N/20 NaOH or HCl[3] as needed. A few drops are usually sufficient.

To the sample add 4 drops more of phenol red and then to both the sample and the color screen tubes add 8 cc. of formalin.[2] Twirl the sample tube just sufficiently to mix the formalin with the sample and, as quickly as possible but with as little agitation as possible, add from a burette[3] N/20 NaOH until the pink color of the indicator is visible, then more deliberately titrate to pH 8.0. After subtracting the titration of the formalin blank the result multiplied by 5 is the formol titration expressed in terms of per cent normal. By multiplying the latter result by 14 the result may be expressed as milligrams of nitrogen per 100 cc. of medium.

The method above described resembles that of Kendall, Day and Walker (1913) and that of Berman and Rettger (1918) in that the sample is brought to a certain hydrogen ion concentration before formalin is added and titrated back to the same hydrogen ion concentration after the addition of the formalin, but it differs from them in other respects. Kendall, Day and Walker allowed the sample of medium to stand thirty minutes after the addition of formalin before proceeding with the titration. We have found this not only unnecessary but distinctly bad because of the loss of CO_2 into the atmosphere. The CO_2 may be removed before the formalin is added in which case any ammonium carbonate which was present is transformed into other ammonium salts and there will be no further loss of CO_2 after the formalin is added, but the escape of CO_2 after the formalin is added means a decreasing titratable acidity and hence lowers the formol titration. The CO_2 must either be removed before adding the formalin or must be kept in solution until the titration is finished. If in the presence of carbonates it is desired to allow the mixture of formalin and sample to stand for a few minutes it should first be brought to an alkaline reaction. The effect of the escape of CO_2 into the air is illustrated by the formol titration curve of ammonium carbonate in Plate 3. This titration was carried out with four different indicators in four different samples of ammonium

[3] The burette used should be finely graduated so that it can be read in hundredths of a cubic centimeter. The N/20 NaOH and HCl used should contain phenol red in the same concentration as is present in the sample and in the colorimetric standards, a suggestion adopted from Hurowitz, Meyer, and Ostenberg (1915).

carbonate solution. Formalin was added to the first sample (methyl red) and it was titrated deliberately from pH 5.2 to 5.6. Formalin was added to a second sample (brom cresol purple) and it was titrated from pH 6.0 to 6.8. Similarly a third sample (phenol red) was titrated from pH 7.0 to 8.4. The fourth sample (thymol blue) was titrated from pH 8.8 to 9.0. It will be noted that as the titration of each sample progressed the curves ending on the acid side of pH 7.0 fell short, so as to produce breaks or steps in the entire curve at A and B, a condition which did not occur in the absence of carbonates, as is illustrated by the curves of the other substances similarly titrated. We attribute these breaks to loss of CO_2. This is further illustrated in Table II.

TABLE II.

Illustrating the Effect of Loss of CO_2 during the Formol Titration.

Sample.	Procedure.	Result.
Glycine	Titrated deliberately.	2.53*
Glycine + 1 per cent $NaHCO_3$	Titrated several minutes after addition of formalin.	2.34
Glycine + 1 per cent $NaHCO_3$	Titrated quickly after addition of formalin.	2.52
Glycine + 1 per cent $NaHCO_3$	Titrated under oil.	2.55
Glycine + 1 per cent NaH_2PO_4	Titrated deliberately.	2.54

* In this and the succeeding tables the results are expressed as cubic centimeters of N/20 NaOH required for the titration of 1 cc. of sample, the formalin blank[2] having been subtracted.

By bringing the reaction back to alkaline as quickly as possible after the addition of formalin the loss of CO_2 into the air is minimized. By adjusting the reaction of the sample to pH 8.0 and carrying the final titration to the same point the buffer effect of phosphates, carbonates, or other buffer substances in the medium is eliminated. That by these means the necessity for removing the phosphates and carbonates by precipitation is obviated is illustrated in Tables II and III. Other means have been tried, such as preliminary acidification and boiling or aeration to get rid of the CO_2, or titration under a layer of oil, but the technic described above is almost if not quite as efficient and much simpler.

From a study of the titration curves of pure amino-acids and ammonium salts already described it was concluded that optimum results were obtained by adjusting the reaction of the sample to pH 6.8 or 7.0 then adding the formalin and titrating to pH 8.0 or 8.4. However, with bouillon, and particularly if phosphates or carbonates have been added, titration between these limits of hydrogen ion concentration is impossible as is also illustrated in Table III. To

TABLE III.

Formol Titrations in the Presence of Carbonates and Phosphates.

Sample.	Initial point of titration.	End point of titration.	Result.
	pH	*pH*	
Bouillon only (containing only the phosphates and carbonates which are native to meat infusion bouillon).	7.0	8.0	1.35
	7.0	8.4	1.35
	8.0	8.0	1.07
	8.4	8.4	0.96
Bouillon + 1 per cent $NaHCO_3$ and 1 per cent Na_2HPO_4 (titrated quickly).	7.0	8.0	1.9
	8.0	8.0	1.06
Bouillon + 1 per cent $NaHCO_3$ and 1 per cent Na_2HPO_4 (carbonates and phosphates precipitated by $BaCl_2$ and filtered out).	7.0	8.0	1.13
Glycine only.	7.0	8.0	1.24
	8.0	8.0	1.22
Glycine + 1 per cent $NaHCO_3$ and 1 per cent Na_2HPO_4 (titrated quickly).	8.0	8.0	1.22
Glycine + 1 per cent $NaHCO_3$ and 1 per cent Na_2HPO_4 (carbonates and phosphates precipitated by $BaCl_2$ and filtered out).	7.0	8.0	1.26

eliminate the buffer effect of these substances the formol titration must start and end at the same hydrogen ion concentration. It is therefore imperative to determine what this hydrogen ion concentration should be. For each of the pure substances titrated formol titrations were made between such limits as pH 5.6 to 5.6, pH 6.8 to 6.8, pH 7.0 to 7.0, pH 7.6 to 7.6, pH 8.0 to 8.0, pH 8.4 to 8.4, and pH 9.0 to 9.0. These titrations are indicated by small triangles

in Plates 1, 2 and 3.[4] The results are in strict agreement with what might be deduced from the character of the other curves plotted. If the isoelectric zone of the amino-acid overlaps a portion of the zone of neutralization of the methylene derivative (resultant curve) (i.e., if the two curves fall nearly vertically at the same hydrogen ion concentration) the formol titration gives practically perfect results when carried out between limits of hydrogen ion concentration within the overlapping portion of these zones. For example the isoelectric zone of glycine (Plate 1) and the zone of neutralization of its methylene derivative (resultant curve) overlap from about pH 6.8 to 8.0 and practically 100 per cent of the glycine may be determined by formol titration from pH 6.8 to 6.8 or from pH 8.0 to 8.0. However on either side of these extremes, as shown by the triangles at pH 5.6, 8.4 and 9.4, lower values are obtained. In the case of alanine there is only slight overlapping of the zones at pH 8.0 to perhaps 8.4. In the case of asparagine, ammonium chloride and ammonium lactate there is overlapping from about pH 6.0 to 7.0. In the case of the other amino-acids titrated there is no actual overlapping of the zones, consequently with these substances all formol titrations which begin and end at the same hydrogen ion concentration are too low, and if we are to use the method under consideration the hydrogen ion concentration at which a maximum titration is obtained must be found. Maximum titration values for certain amino-acids were obtained at the following hydrogen ion concentrations; phenylalanine, pH 7.6; tyrosine, pH 8.0; aspartic acid, pH 8.4; glutamic acid, pH 8.0 to 8.4. We did not have the difficulty experienced by Sörensen (1907) (1908) of obtaining results that were too high in the formol titration of tyrosine. With ammonium phosphate and ammonium carbonate another phenomenon is encountered; the line of the formol titrations starting and ending at the same hydrogen ion concentration crosses the titration curves

[4] For all of the solutions, the titrations of which are plotted in Plates 1, 2 and 3, total nitrogen or ammonia determinations were made. On the assumption that these determinations represent 100 per cent of the substance in solution the curves are so adjusted that a perfect formol titration (*i.e.*, titration of 100 per cent of the substance) should require 2 cc. of N/20 NaOH. A solution of this concentration would be one-tenth normal.

of phosphoric and carbonic acids (resultant curves). The formol titration of ammonium phosphate at points to the acid side of pH 7.8 leads to results which are too high while titration at points to the alkaline side of pH 8.0 gives low results. Perfect results are obtained at pH 7.8 or 8.0. Formol titration of ammonium carbonate did not yield 100 per cent of the ammonium value probably because of the escape of some of the CO_2 during titration, but maximum results (at least 90 per cent of the theoretical) were obtained at pH 6.0, 7.0, or 8.0.

TABLE IV.

Formol Titrations of Bacteriological Media at Various Hydrogen Ion Concentrations.

Medium.	The initial and end points of titration.				
	pH 7.2–7.2	pH 7.6–7.6	pH 8.0–8.0	pH 8.4–8.4	pH 9.0–9.0
1 per cent beef aminoids*	0.86	0.9	0.95	0.79	0.64
1 per cent casein aminoids*	0.68	0.71	0.74	0.65	0.36
1 per cent Fairchild peptone	0.68	0.67	0.68	0.52	0.39
	(pH 7.0–7.0)				
Veal bouillon 1502	1.04	1.12	1.11	1.04	
Veal bouillon 1502 + 1 per cent NaH_2PO_4	1.35	1.23	1.10	1.04	
Veal bouillon 1502 + 1 per cent $NaHCO_3$	0.81	1.04	1.07	0.99	

* Arlington Chemical Co. product.

If it is desired to perform a formol titration of a mixture of amino-acids and ammonium salts from a selected hydrogen ion concentration back to the same hydrogen ion concentration, as here proposed, the optimum hydrogen ion concentration will evidently depend upon the relative proportions of the various amino-acids and ammonium salts present. This optimum hydrogen ion concentration has been determined empirically for solutions of "aminoids," peptone and standard meat infusion bouillon (see Table IV).

The "aminoids" gave maximum formol titrations at pH 8.0. Fairchild peptone gave maximum results between pH 7.2 and pH 8.0. The veal infusion bouillon gave maximum titrations of pH 7.6 and pH 8.0. In the case of other samples of bouillon we have found the maximum more often at pH 8.0 than at pH 7.6. When $NaHCO_3$

or NaH_2PO_4 was added to the bouillon practically the same result was obtained at pH 8.0, but in the phosphate bouillon higher titrations were obtained at pH 7.6 and at pH 7.0. This phenomenon is related to a similar peculiarity of the ammonium phosphate curve (Plate 3) already commented upon. This fact and the comparison with the titrations of the plain bouillon and other substances serve to indicate that the formol titration of bouillon from pH 8.0 to 8.0 gives the most nearly correct value, a value very close to that which may be obtained after removal of the phosphates and carbonates. In the case of pure amino-acids and ammonium salts the formol

TABLE V.

Comparison of the Results of Formol Titration by the Methods of Kendall, Berman and Rettger, and the Method Here Described.

Sample.	Methods of		
	Kendall.	Berman and Rettger.	The author.
Glycine	2.27*		2.58
Asparagine	0.86		1.11
Veal bouillon	0.86	0.65	1.07

* For purpose of comparison all the results are reduced to the same terms. The figures represent cc. of N/20 NaOH required for the titration of each 1 cc. of sample, the formalin blank[2] having been subtracted in the author's method, the formalin having been "neutralized" to phenolphthalein in the methods of Kendall and of Berman and Rettger.

titration from pH 8.0 to 8.0 yields from 90 to 100 per cent of the theoretical value, depending upon the individual substance titrated.[5]

It will be of interest to know how the results so obtained compare with those obtained by the methods of Kendall, Day and Walker (1913), Foster and Randall (1921), Kendall (1922), and Berman and Rettger (1918). The results recorded in Table V serve to show

[5] Eckweiler, Noyes and Falk (1921) have published a titration curve of glycylglycine which is said to be similar to that of other simple dipeptides. It is to be noted that according to this curve the formol titration of these substances from pH 8.0 to 8.0 would introduce a larger error than that found in the titration of simple amino-acids. However, in the case of standard meat infusion bouillon at least, the sum of errors is not a large one.

that the results of Kendall's method are probably at least 20 per cent too low and those of Berman and Rettger even lower. Assuming that the ammonia determinations in the work of these authors were correct it will be seen that when the ammonia nitrogen was subtracted from the formol nitrogen the resulting amino nitrogen value probably involved an error even greater than that indicated by the formol titration alone. Three factors must have conspired to produce these errors: (1) the use of too much water and too little formalin, (2) the titration from pH 8.3 or 8.4 (the end point of phenol-

TABLE VI.

Formol Titration of Mixtures of an Amino-Acid and an Ammonium Salt.

Sample.	Formol titration (pH 8.0 to 8.0).
1 cc. glycine solution	2.53
1 cc. ammonium lactate solution	1.37
Sum	3.90
Mixture of 1 cc. glycine solution and 1 cc. ammonium lactate solution	3.91

TABLE VII.

Formol Titration of an Amino-Acid Added to Bouillon.

Sample.	Formol titration (pH 8.0 to 8.0).
1 cc. glycine solution	1.17
1 cc. bouillon	1.09
Sum	2.26
Mixture of 1 cc. glycine solution and 1 cc. bouillon	2.26

phthalein) back to pH 8.3 or 8.4, and (3) the loss of CO_2 from the sample after formalin had been added.

In criticism of the method of Sörensen it was claimed by de Jager (1909) that a mixture of glycine and ammonium salt gave a formol titration which was lower than the sum of the formol titrations of the glycine and ammonium salt made separately. Henriques and Sörensen (1910) found this of little consequence in the titration of urines. In Table VI it is shown that this is not a disturbing factor in the method here described.

In the growth of bacterial cultures amino-acids are often liberated from protein substances in the medium. It is therefore of direct

interest to know whether the amount of such acid liberated may be actually measured by the formol titration. The formol titration of bouillon before and after the addition of a titrated amount of glycine indicates that it can be so measured (see Table VII).

SUMMARY.

The influence of certain factors involved in the formol titration of amino-acids and ammonium salts is illustrated. Their titration curves are plotted.

Serious errors in current methods of performing the formol titration of bacteriological media are pointed out.

It is shown that the formol titration of bacteriological media presents a special problem. Simple methods of performing this titration are described, methods requiring very small samples of media. Method A is a modification of the technic of Henriques and Sörensen for the formol titration of urine and involves the removal of carbonates and phosphates by precipitation with barium. Method B is almost as accurate as A, yielding results which probably represent 94 per cent of the amino-acids and ammonia present, and does not require the removal of carbonates and phosphates.

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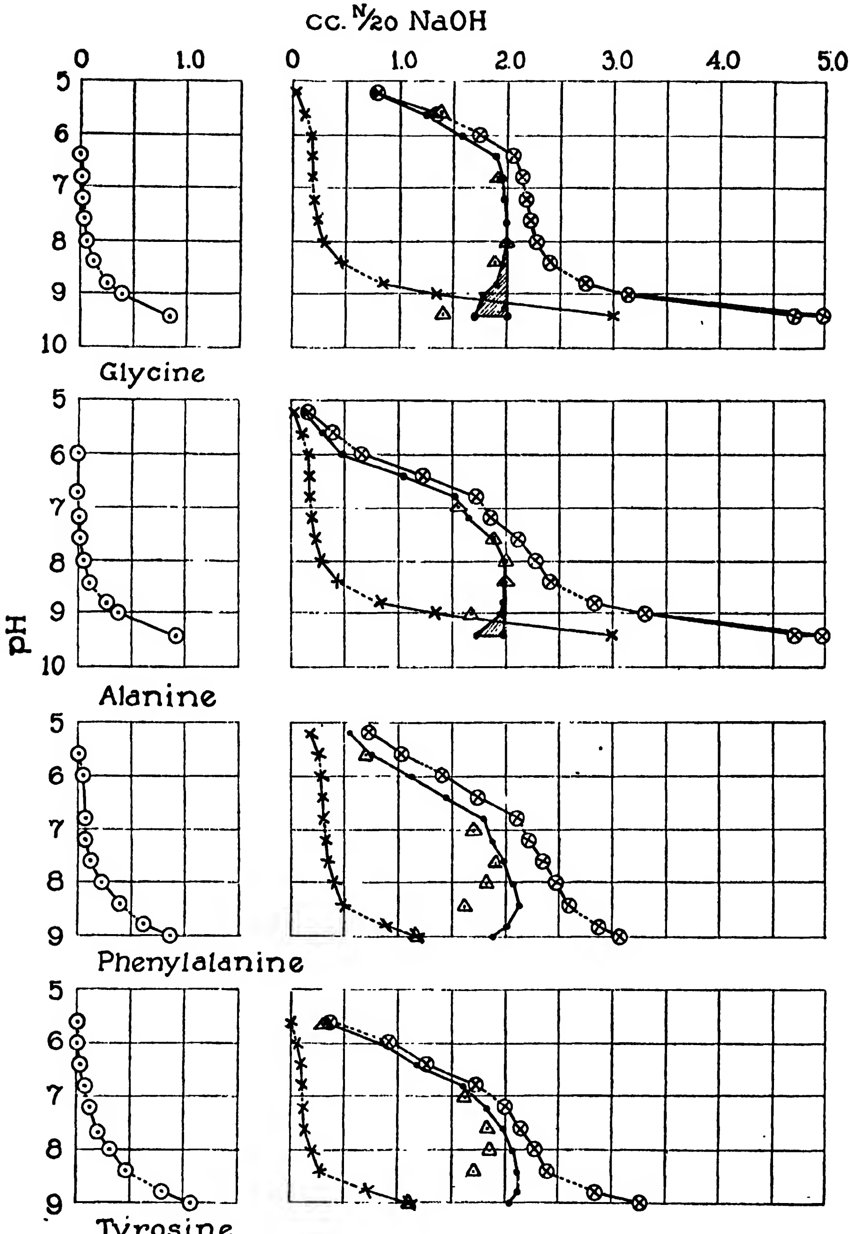
EXPLANATION OF PLATES.

1. —•—•— titration curve of 1 cc. of sample plus 9 cc. water.
2. —x—x— titration curve of 8 cc. formalin plus 10 cc. water.
3. —•—•— titration curve of 1 cc. sample plus 9 cc. water and 8 cc. formalin.
4. —•—•— resultant curve obtained by subtracting values on curve 2 from those on curve 3.
5. Δ formol titrations.

Note: The substance titrated, i.e., the sample, is indicated by the name printed beneath each set of curves.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0
1.0
0
1.0
2.0
3.0
4.0
5.0
pH
5
6
7
8
9
10
Glycine
Alanine
Phenylalanine
Tyrosine

PLATE 1.

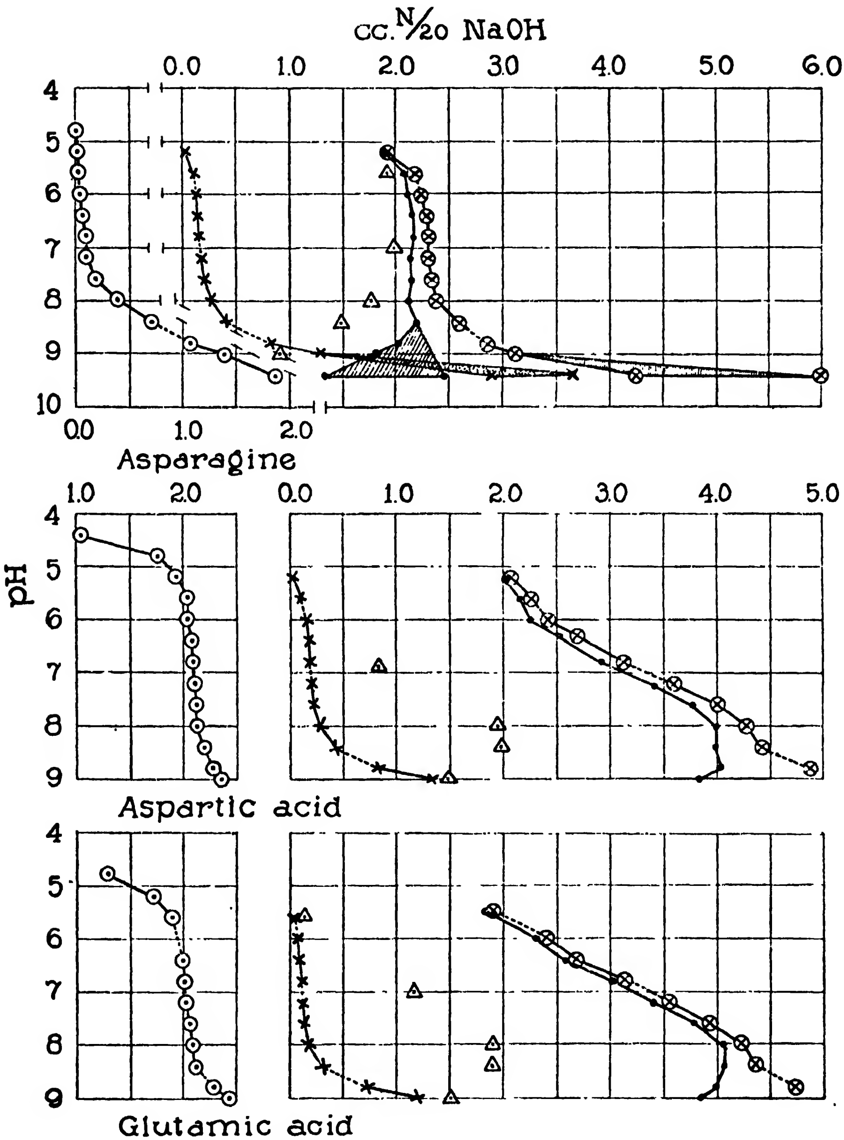
cc. N/20 NaOH
0.0
1.0
2.0
3.0
4.0
5.0
6.0
4
5
6
7
8
9
10
0.0
1.0
2.0
Asparagine
1.0
2.0
0.0
1.0
2.0
3.0
4.0
5.0
pH
4
5
6
7
8
9
Aspartic acid
4
5
6
7
8
9
Glutamic acid

PLATE 2.

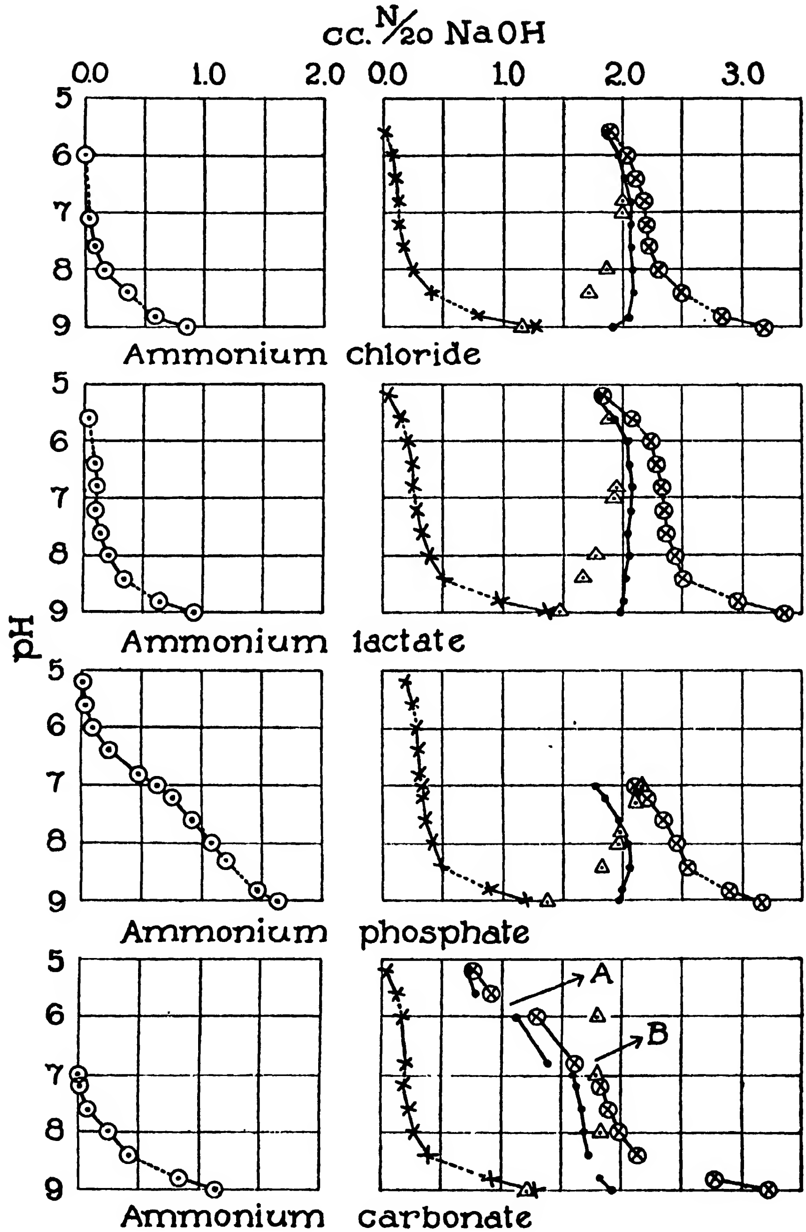
cc. N/20 NaOH
0.0
1.0
2.0
0.0
1.0
2.0
3.0
pH
5
6
7
8
9
Ammonium chloride
Ammonium lactate
Ammonium phosphate
Ammonium carbonate
A
B

PLATE 3.

[Reprinted from The Journal of Experimental Medicine, June 1, 1923, Vol. xxxvii, No. 6, pp. 789–798.]

THE PRODUCTION OF ANTIBODIES IN RABBITS BY A SIMPLIFIED INTRATRACHEAL METHOD.

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(Received for publication, January 27, 1923.)

During the last few years interest has been revived in antibody production by means of injections into the trachea. Besredka[1] called attention to the laryngotracheal method of administering sera to guinea pigs. Among other experiments two guinea pigs were vaccinated with killed cultures of the diphtheria bacillus. One was treated intratracheally, the other inoculated into the peritoneal cavity. 11 days after the last injection each received an intratracheal injection of living culture. The animal that had been vaccinated by the intratracheal route survived; the other died. Both animals had developed agglutinins for the diphtheria organism.

Pfenninger[2] undertook a more detailed study of antibody production after the administration of antigens through the trachea. He incised the skin overlying the trachea and injected through a needle inserted between the cartilaginous rings. The injections were made 7 days apart. Comparisons were made of the intravenous, the intraperitoneal, and the intratracheal methods. Pfenninger pointed out that the intratracheal method for antibody production is a little better on the whole than the intravenous route. He considered both superior to the peritoneal route. Many of his rabbits died after several intravascular inoculations, but all those treated by the tracheal and peritoneal methods survived.

D'Aunoy[3] used much the same procedure. He concludes that the intratracheal injection is comparable with intravenous inoculation and superior to intraperitoneal injection for the production of agglutinins, bacteriolysins, bactericidins, and precipitins. Hemolysins were formed more slowly by intratracheal administration but ultimately the serum obtained was equal to that produced in the intravenous series. Attention is directed to the relative safety of the intratracheal method, since none of the rabbits died during the experiments. Several deaths occurred in the intravenous series.

A simple method for the administration of liquids into the trachea seemed desirable, preferably one which would produce the minimum amount of injury and

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[1] Besredka, A., *Ann. Inst. Pasteur*, 1920, xxxiv, 361.

[2] Pfenninger, W., *Ann. Inst. Pasteur*, 1921, xxxv, 237.

[3] D'Aunoy, R., *J. Infect. Dis.*, 1922, xxx, 347.

enable the operator to inject animals rapidly. Factors other than the introduction of antigen into the respiratory tract have not been taken into account when injections through the walls of the trachea have been made. Winternitz, Smith, and Robinson[4] point out that injection of material through the walls of the trachea affords an opportunity for infection of the submucosa and the peritracheal tissue. They observed histological evidences of infection and were able to trace the injected organisms through the submucosa of the trachea and larger bronchi to the hilus of the lung by way of the peribronchial and perivascular structures.

Snel[5] had previously shown by experiments with guinea pigs that the anthrax bacillus failed to attack the intact lung. When the vegetative forms or spores were introduced by means of a catheter passed through the larynx, infection failed to take place. In guinea pigs injected by means of a needle inserted through the walls of the trachea a locus of infection was produced and the animals developed a septicemia.

Kitt[6] also cites similar observations by Arloing, Cornevin, and Thomas in immunization against blackleg. They showed that following the introduction of virulent material into the veins or the tracheæ, calves resisted subsequent subcutaneous or intramuscular injections. Great care was employed to avoid leakage into the perivascular and peritracheal tissues since the virus would multiply in these structures with fatal results.

An apparatus by which one could introduce fluids into the lower respiratory tract without appreciable injury to the mucosa and underlying structures would imitate more nearly the natural condition. In addition, a method which would enable one to space the doses at frequent intervals would be of considerable advantage.

Woven or rubber catheters introduced through the glottis have been employed by many for intratracheal inoculation, but considerable skill is required in their use.

Method of Intratracheal Injection.

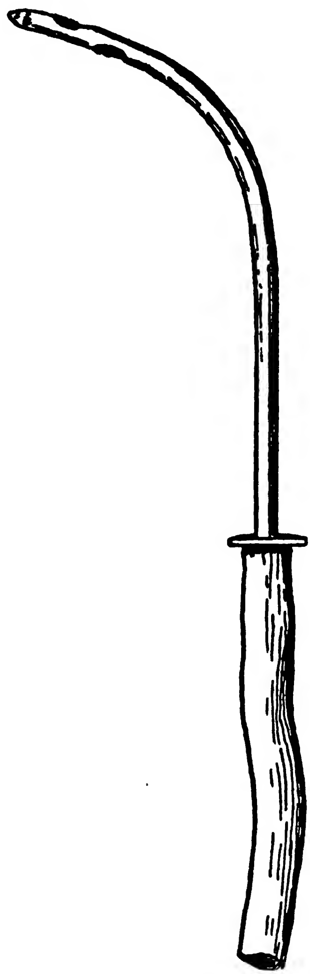
It has been stated that as simple a device as possible seemed advisable. It was found that a metal tube with a rounded end could be easily introduced into the trachea through the glottis. A milk or teat tube 9 cm. long, with an external diameter of 3 mm., was bent to a final angle of about 70° (Text-fig. 1). Such a tube has two open-

[4] Winternitz, M. C., Smith, G. H., and Robinson, E. S., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 195.

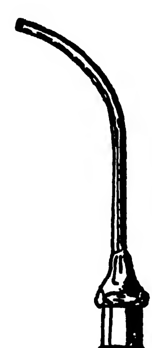
[5] Snel, J. J., *Z. Hyg. u. Infectionskrankh.*, 1902, xl, 103.

[6] Kitt, T., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1912, iv, 819.

ings on opposite sides situated a short distance from the end so that very little force is required to inject fluids. Rabbits are given sufficient ether to insure complete relaxation. The mouth is opened and the tongue grasped gently by means of rubber forceps and drawn



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. Tube for the administration of liquids into the trachea of rabbits. Actual size.

TEXT-FIG. 2. Tube for the administration of liquids into the trachea of guinea pigs. Actual size.

forward. The tube, previously immersed in paraffin oil, is inserted into the mouth and carried backward over the tongue and downward through the larynx. The tube is then connected to a syringe and the fluid slowly forced into the trachea. The tube is retained for a few seconds to allow drainage and then slowly withdrawn. After a little practise the whole operation may be done with considerable rapidity —usually from 1½ to 3 minutes elapse between the beginning of anesthesia and the completion of the injection. The anesthesia may be so controlled that the animal has almost recovered when the tube is withdrawn.

A similar tube made from a 2½ inch 14 gauge hypodermic needle will answer for guinea pigs (Text-fig. 2). Obviously the operation is more difficult in the guinea pig on account of the small mouth and the great development of the tongue at its base. A properly directed light is of considerable value when guinea pigs are used.

Comparison of the Production of Antibodies in Rabbits by Means of the Intratracheal and Intraperitoneal Routes.

Throughout the experiments the rabbits were immunized in pairs. The dose of antigen was always the same; the volume never exceeded 1.5 cc. In the case of bacterial and red cell suspensions the amount of material was increased from one series to another by concentration. Small volumes were indicated, since it was felt that the likelihood of all the material being retained in the respiratory tract was greater. All injections were made in series. Injections were made daily for 3 days, and 5 days after the last injection the animals were bled and the next series begun. The rabbits subjected to the peritoneal treatment were anesthetized for the same length of time as those injected into the trachea. Inasmuch as the protocols add little of interest, an outline of one is given in Table I.

In these experiments a heavy suspension in salt solution from a 24 hour agar culture of the hog-cholera bacillus was killed by heating to 60°C. for ½ hour. This suspension was stored in the refrigerator as the stock vaccine. From it the antigens for the various series were prepared from week to week.

The results obtained in the case of these two rabbits have been recorded in Table II.

TABLE I.

Protocols of Rabbits Treated Intratracheally and Intraperitoneally with Killed Cultures of the Hog-Cholera Bacillus.

Rabbit No.	Method of administration.*	Series No.	Turbidity of antigen by Gates apparatus.	Time elapsing between beginning of anesthesia and completion of injection.	Thermic reaction.
				min.	
1	Intratracheal.	1	1.0	1½	None.
				2	Severe.
				1¼	"
2	Intraperitoneal.	1	1.0	1½	Slight.
				2	None.
				1½	"
1	Intratracheal.	2	0.8	2	Moderate.
				1½	"
				2	"
2	Intraperitoneal.	2	0.8	2	Slight.
				1½	"
				6	None.
1	Intratracheal.	3	0.5	2	Moderate.
				2	Severe.
				2	Moderate.
2	Intraperitoneal.	3	0.5	2	None.
				2	Slight.
				2	None.
1	Intratracheal.	4	0.4	2	Severe.
				2	Moderate.
				2	"
2	Intraperitoneal.	4	0.4	2	None.
				2	"
				2	"
1	Intratracheal.	5	0.3	2	Moderate.
				2	Severe.
				2	"
2	Intraperitoneal.	5	0.3	2	Slight.
				2	None.
				2	"

* The dose was always 1 cc.

TABLE II.

Agglutinin Production in Rabbits Immunized by the Intratracheal and Intraperitoneal Routes.

Rabbit No.	Method of administration.	Time.	Serum dilutions.													
			1:5	1:10	1:20	1:50	1:100	1:200	1:500	1:1,000	1:2,000	1:5,000	1:10,000	1:20,000	1:50,000	1:100,000
		wks.														
1	Intratracheal.	1	C.*	C.	C.	++	+	±	±	−	−					
2	Intraperitoneal.	1	"	"	+++	+	+	+	±	−	−					
1	Intratracheal.	2	"	"	C.	C.	C.	C.	++++	++	+	±	−			
2	Intraperitoneal.	2	"	"	"	"	"	"	++++	+++	+	±	−			
1	Intratracheal.	3			"	"	"	"	C.	C.	++++	+++	++	+	−	−
2	Intraperitoneal.	3			"	"	"	"	"	"	+++	+++	++	+	±	−
1	Intratracheal.	4				"	"	"	"	"	++++	+++	++	++	+	±
2	Intraperitoneal.	4				"	"	"	"	"	C.	++++	+++	++	±	−
1	Intratracheal.	5					"	"	"	"	++++	+++	++	+	±	−
2	Intraperitoneal.	5					"	"	"	"	C.	++++	+++	++	+	±

* The readings were recorded as follows: C., complete clearing of the fluid; ++++, heavy deposit with slight turbidity of the liquid; +++, less marked agglutination; ++, moderate clumping; +, distinct deposit; and ±, slight deposit.

From the tables it will be noted that the temperature reactions following the intratracheal inoculation are on the whole more severe. The temperature of animals that have been vaccinated through the tracheal route usually falls 1°C. within an hour or two. This is followed by a rather sharp rise for several hours. The initial drop in temperature after the intraperitoneal administration is much less and the secondary increase is not so pronounced. After 3 or 4 weeks of treatment those animals injected intratracheally with bacterial vaccines usually develop a severe dyspnea immediately after the treatment. In two rabbits treated for considerable periods consolidation associated with *Bacillus lepisepticus* has been observed.

It will be noted that agglutinins developed to about the same degree in both animals. This has held true in experiments with other organisms.

Similar results have been obtained in the production of hemolysins and precipitins. For the production of a hemolytic serum rabbits were immunized with a suspension of the washed red cells of the sheep. As in the preceding experiments the amount of material injected at one time was limited to 1 cc. The animals received three series of weekly injections. The first series contained a 10 per cent suspension of red cells, the second a 20 per cent suspension, and in the third the cells were increased to 40 per cent. In this way constantly increasing doses of antigen were possible. The total number given was equivalent to 42 cc. of a 5 per cent suspension of sheep red cells. The results are given in Table III.

The rabbit treated by the intratracheal route formed hemolysin to a greater degree during the first series of inoculations than the other which received intraperitoneal injections. After the second series the animal injected intraperitoneally possessed the better serum. At the end of the third series the titer of both sera was about the same.

It was possible to produce a good precipitating serum in the same manner. In these experiments cow serum was administered by both routes. The maximum dose given at one time was 1.5 cc. A total of 12 cc. was administered over a period of 3 weeks. The results are recorded in Table IV. In more recent work it was found that 6 to 8 cc. of a foreign serum may be introduced into the trachea without causing noticeable ill effects.

TABLE III.

Hemolysin Production in Rabbits Immunized by the Intratracheal and Intraperitoneal Routes.

Rabbit No.	Method of administration.	Time.	Serum dilutions.										
			1:5	1:10	1:20	1:50	1:100	1:200	1:500	1:1,000	1:2,000	1:5,000	1:10,000
		wks.											
3	Intratracheal.	1	C.*	C.	++++	+++	++	+	±	±			
4	Intraperitoneal.	1	"	++++	++	+	±	–	–	–			
3	Intratracheal.	2	"	C.	C.	C.	C.	C.	C.	C.	+++	+	–
4	Intraperitoneal.	2	"	"	"	"	"	"	++++	+++	±	±	–
3	Intratracheal.	3			"	"	"	"	C.	C.	++++	+	–
4	Intraperitoneal.	3			"	"	"	"	"	"	++++	++	±

* The usual method of recording hemolysis has been employed. The serum was stored in the refrigerator from 2 to 4 weeks before it was tested. Fresh guinea pig serum was used for complement.

During the first 2 weeks there was considerable difference in the antibody content of the two sera. At the end of the 1st week the serum of the rabbit (No. 5) immunized by intratracheal inoculation detected 1/1,000 cc. of cow serum; that of the other (No. 6) failed to react in the presence of 1/100 cc. By the end of the 2nd week the serum of Rabbit 5 had almost reached its maximum titer and reacted slightly with 1/20,000 cc. of cow serum. After 2 weeks 1/2,000 cc. of antigen was required to produce precipitation with the serum of Rabbit 6. At the end of the 3rd week both sera were equally efficient.

TABLE IV.

Precipitin Production in Rabbits Immunized with Beef Serum by Injection into the Tracheal and Peritoneal Cavities.

Rabbit No.	Method of administration.	Time.	Amount of antigen (in cc.) producing precipitation with 0.1 cc. of serum.*									
			1/100	1/200	1/500	1/1,000	1/2,000	1/5,000	1/10,000	1/20,000	1/50,000	1/100,000
		wks.										
5	Intratracheal.	1	+	+	+	+	±	−	−	−		
6	Intraperitoneal.	1	±	±	−	−	−	−	−	−		
5	Intratracheal.	2	+	+	+	+	+	+	+	±		
6	Intraperitoneal.	2	+	+	+	+	+	−	−	−		
5	Intratracheal.	3	+	+	+	+	+	+	+	+	±	−
6	Intraperitoneal.	3	+	+	+	+	+	+	+	+	−	−

* Definite precipitation has been recorded as +; ± indicates a slight turbidity and a trace of sediment.

DISCUSSION AND SUMMARY.

The method described of producing antibodies by the administration of antigens through the larynx is simple. The results obtained, however, conform closely to those obtained through intraperitoneal injection. The procedure is relatively a safe one and may well be employed in experimental inoculations. The advantages of rapidity and painlessness are obvious. In addition, gross injury has not been observed. Injections may be repeated at frequent intervals without danger to the life of the animal. The tube illustrated in Text-fig. 1

extends a little over 1 cm. into the trachea. The tube designed for the guinea pig reaches 2 or 3 mm. below the glottis. While the doses indicated in the protocols are small, 7 to 10 cc. of liquid have been given to rabbits by means of the tube without ill effects. It has been shown by the injection of India ink that the material is well distributed throughout the lungs in both the rabbit and guinea pig.

Under certain conditions it may be advisable to inject more deeply into the trachea, especially in the rabbit. The tube illustrated in Text-fig. 1 is not adaptable for this purpose. A cannula of larger diameter has proved of distinct advantage as a shield for introducing flexible catheters well down the trachea. A cannula 9 cm. long and 4 mm. in diameter when bent at an angle of 45° may be passed through the larynx of a rabbit without difficulty. A No. 8 (French) woven or No. 10 soft rubber catheter may be inserted into the cannula beyond the bend before the tube is passed through the glottis. After the tube has entered the glottis the catheter then may be introduced as deeply as desired into the teachea. The metal cannula enables the operator to "feel" the glottis.

From the experiments one seems justified in concluding that the results obtained by administering antigens by way of the trachea are about the same as those obtained by the intraperitoneal route. With the tubes described injury has been largely eliminated. The procedure is relatively a safe one.

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MUTATION AMONG HOG-CHOLERA BACILLI.

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(Received for publication, February 23, 1923.)

Mutations of various kinds have been demonstrated to occur among bacteria. Among the early workers Baerthlein[1] made an extensive study of many strains including cholera vibrios, typhoid, paratyphoid, hog-cholera, and dysentery bacilli, *B. coli*, *B. enteritidis*, *B. alkaligenes*, *B. prodigiosus*, *B. pyocyaneus*, and streptococci. He described changes or mutations concerning various characters. as colony formation, morphology, cultural reactions, fermentation powers, hemolysis, pigment production, and agglutinability. He also has given a review of much of the work on mutation up to that time. Baerthlein's description of mutation of the *B. suipestifer* (hog-cholera) strains is of special interest in connection with what follows. From hog-cholera cultures he obtained three groups of mutants according to colony formation. He stated that the first appearances of mutation were found after the culture had been kept for a long time (2 to 3 months) on agar without transplanting.

Among the more recent workers, Arkwright[2] described smooth and granular forms among Shiga dysentery cultures which were similar to the hog-cholera types reported in this paper. Arkwright gave quite an extensive review of the earlier literature on mutation. His own work was with the colon-typhoid group and especially with the Shiga bacilli. He reported an S and an R form arising from a single strain which remained constant in weekly subcultures in bouillon. The S form produced smooth colonies, uniform clouding of bouillon, and stable suspension in salt solution. The R form gave granular colonies, grew as a deposit in bouillon, and agglutinated spontaneously in salt solution. He was able to produce specific sera and found the S types of all strains serologically alike but different from the R types and the R forms like each other but different from the S. He found no difference in the virulence of the two types.

Ishii[3] also studied the colon-typhoid group and reported spontaneously and normally agglutinating types. He found connected with these characters smooth colonies and even clouding of bouillon associated with the normally agglutinating

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[1] Baerthlein, K., *Arb. k. Gsndhtsamte*, 1912, xl, 433.

[2] Arkwright, J. A., *J. Path. and Bact.*, 1921, xxiv, 36.

[3] Ishii, O., *J. Bact.*, 1922, vii, 71.

type and granular colonies and clumping in bouillon associated with the spontaneously agglutinating type.

De Kruif[4-6] found the smooth and granular types in cultures of the rabbit septicemia bacillus, which he designated D and G types. He showed that the G form was derived from the D. He distinguished it as a mutant by its acid agglutination optimum. He also found a considerable difference in the virulence of these two forms. Apparently the granular type of mutation is widely distributed among various groups of organisms.

In the following pages are described observations made on a number of strains of *Bacillus suipestifer*, the true hog-cholera bacillus, taken from a collection of which many were isolated and tested by Dr. Theobald Smith. Owing to the fact that this subject is not new and that the methods of work have been fairly well standardized most details of the experiments are omitted and only the results briefly given.

Most of the work to be reported was done upon Strain I isolated in 1898 and kept on ordinary nutrient agar. During the course of some agglutination tests this strain was found to agglutinate spontaneously in normal salt solution. A bouillon culture of this strain was clouded and also gave considerable sediment. When it was plated on agar two different types of colonies were found. When cultures were isolated from separate colonies it was found that the even, smooth colony produced uniform clouding in bouillon with no sediment and did not agglutinate spontaneously in normal salt solution, while the more irregular, granular colony grew in bouillon as a sediment and agglutinated spontaneously in salt solution. These two types have been designated A and B; A for the original smooth type and B for the granular mutant.

Later a moist, opaque colony was isolated from the A type culture and designated A-v. Also in an old bouillon culture of the B type mutant a very small colony (B-v) was found which otherwise possessed the B characteristics. These granular and opaque growths in the individual strain are considered mutations since they can be separated from individual colonies into distinct strains which keep

[4] De Kruif, P. H., *J. Exp. Med.*, 1922, xxxv, 561.

[5] De Kruif, P. H., *J. Exp. Med.*, 1921, xxxiii, 773.

[6] De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 387.

their particular granular or opaque characters through successive transplants.

An examination of twelve other stock cultures consisting of both old and young strains showed the same two types of growth present in seven of these cultures, three of which were rather recently isolated. One older strain gave apparently a pure granular type, while another old culture showed a mixed appearance but the individual types were not separated. In the three remaining strains no granular growth has been detected. This mutation into the granular type does not depend entirely upon the age of the strain but seems to be associated with the length of time a given transplant is kept before it is renewed.

After it was established that two types of growth existed in the hog-cholera strains, experiments were made with Strain I to determine the constancy of each type in pure culture. First the constancy of the types kept on agar and stored in the refrigerator was tested. The second series of experiments was made with bouillon cultures kept at 37°C. and at room temperature. The third group of experiments was made to test the effect of rapid successive passages through bouillon on the constancy of the type. The method of examination was the same as that used by other workers; *i.e.*, at frequent intervals the cultures were streaked on agar plates and the colony formation examined. These observations were controlled by determining the character of the bouillon growth of the individual colonies.

In general, the results indicated that the older A cultures, isolated from a single colony to begin with, if kept long enough without transplanting finally showed B type mutants, but if transplanted often they kept their A form. On the other hand, the mutant B type remained true to type with no reversion, except under the conditions of rapid successive passages through bouillon where it tended to resume some of the original characters. Apparently active growth stimulated A type formation, while aging without transplanting caused mutation to the B type. These observations agree only in part with those of Baerthlein.[1] Whereas he found that his original culture mutated, after standing, into two types, and that these two isolated types both remained true by frequent transplanting, and both changed again on standing, we found that this occurred only with the original culture and its corresponding isolated type (A type),

while with the other mutant (B type) the reverse occurred; *i.e.*, the granular mutant remained true to type when kept 4 to 6 weeks or longer without transplanting but very frequent successive passage through bouillon caused a reappearance of some of the original characters.

Another point mentioned by Baerthlein[7] and De Kruif[4] was an increase followed by a decrease in the number of mutants present in a given culture. This rise and fall in the number of mutants was also observed here with the bouillon cultures of the hog-cholera Strain I kept at 37°C. and at room temperature.

To demonstrate further that B is a mutant with characters distinct from A, the morphology and cultural characters were studied. The behavior in different concentrations of NaCl solution, the acid agglutination, and the behavior in different dilutions of normal serum were tested. Also the virulence of the two types for mice was determined. With the exception of the experiments on virulence, which were carried out with three strains only, these tests were made with the A and B types of six strains and A-v and B-v forms of Strain I. These six strains which have been kept on ordinary agar represent cultures of different ages as indicated by the following dates of isolation: Strains I (1898), II (1921), III (1919), IV (1899), V (1898), and VI (1886).

Description of Types.

Morphology.—The types were similar in form and size. They stained as small, short, Gram-negative rods with a few slightly larger forms often seen in both types. The A type always occurred as single, scattered rods, while the B type usually occurred in clumps. Both types were motile. With Strain I the A type apparently was more active than the B type.

Cultural Characters.—The growth of A and B types on agar and in bouillon has already been described for Strain I and is the same in all the other strains. The A-v mutant was a moist, opaque, non-viscid colony, smooth, glistening, round, and yellowish white. It gave an even clouding in bouillon like the A type. The B-v form

[7] Baerthlein, K., *Centr. Bakt.*, *1te Abt.*, *Orig.*, 1913, lxxi, 1.

was a small colony, round but more or less granular under a low power. In bouillon it grew as fine suspended particles and sediment.

Salt Solution Agglutination Reactions.—The difference in the reaction of the two types in 0.85 per cent NaCl solution was marked. The A type gave an even, stable suspension while the B type was completely agglutinated, settling out as a sediment and leaving the fluid clear. By diluting the salt solution with distilled water the spontaneous clumping occurred to a smaller degree. The lower the per cent of NaCl the less the clumping of the B type. The same reaction was seen in bouillon. In standard bouillon the B type grew as a sediment with no clouding but if the bouillon was diluted 1:4 with distilled water the B type grew with a fair amount of clouding. This same reaction was reported by Arkwright[2] in his study of the S and R forms of the Shiga dysentery bacilli. Also the A-v and B-v forms agree with the A and B types in the salt solution agglutination reactions.

Acid Agglutination Reactions.—De Kruif[6] considered a change in the acid agglutination optimum to be an index of bacterial mutation. In comparing the acid agglutination reactions of the hog-cholera types it was found that they gave distinctly different titrations. In making the acid tests the pH values were obtained from KH phthalate-NaOH, KH phthalate-HCl, and KCl-HCl mixtures as given by Clark and Lubs.[8] This series gave a long range of pH values. The cultures were grown on agar for 24 hours and the growth suspended in distilled water. The cultures were washed twice and then suspended in a third lot of distilled water and diluted in the usual way to the standard density, 2.4 cm. with the Gates instrument.[9] This washing prevented any spontaneous clumping of the B type. The A type, or original form, gave a low reaction between pH 3.2 and 1.6, but it was never complete. The B type showed a somewhat longer range, between pH 4.0 and 1.0, and a much stronger reaction with complete clumping from pH 3.2 to 1.8. The A-v and B-v forms agree with the A and B types respectively in their acid agglutination reactions. The A types of all strains reacted alike and also the B types of all strains.

[8] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1.
[9] Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.

Normal Agglutination Reactions.—The reactions in normal serum were very different for the two types. An attempt was made to prepare specific A and B sera from rabbits, but it was found that the normal rabbit serum agglutinated the B type to a considerable extent in the case of the two rabbits chosen. To determine whether this was true for normal serum in general, the blood of ten normal rabbits and of three guinea pigs was tested. The tests were made so that in the final dilutions the concentration of the NaCl was only 0.1 per cent; thus the clumping of the B type by salt solution was prevented. The cultures were also washed in distilled water twice before making up to the standard density for the tests. The results showed that normal rabbit serum agglutinated A type only in the low dilutions but agglutinated B type completely at 1:320 or 1:640. With normal guinea pig serum the same was true. Thus normal serum agglutinates B type strongly but A type only slightly. These results again showed the A and B forms as distinct types. However, by agglutinin absorption tests the A and B types are found to be related. A normal rabbit serum which agglutinated A at 1:320 and B at 1:1,280, after absorption with A gave an agglutination reaction negative for A and slight at 1:160 for B and exactly the same results after absorption with B. Thus by absorption of a normal serum with the A type the B agglutinins were also removed, and absorption with the B type removed the A agglutinins as well.

Virulence Tests.—The virulence of A and B types was tested by injecting a 20 hour bouillon growth into mice. Three different strains, Nos. I, II, and III, were used. Strain I is very old, but from the subcutaneous inoculation, although the mice did not die, the culture results indicated a difference in virulence. Cultures were made from the mice by inoculating bits of spleen onto agar slants. The tubes inoculated from mice injected with A type culture all gave positive growth, while some of the tubes inoculated from mice injected with B type culture showed growth but others remained sterile, indicating that the A type culture persisted in the mice while the B type culture was destroyed more readily. By intraperitoneal inoculation of this strain it was found that the A type would kill mice while the same dose of the B type did not. Certain irregularities were noticed but these probably were due to differences in the original

resistance of the mice. With the two younger strains, Nos. II and III, the same difference was indicated in a more definite manner. A given dose (0.2 or 0.05 cc. according to the strain) subcutaneously of A type killed the mice, while the same dose of B type caused no disturbance. Here, too, some irregularities occurred but the differences between the types were striking enough to indicate an appreciable difference in virulence in favor of the A type.

CONCLUSIONS.

1. In the hog-cholera group the mutation from smooth (A) to granular (B) type occurs and a mutant with a moist, opaque colony formation was noted.
2. The B mutant when obtained pure remains a B type without reversion in the stock cultures.
3. The B mutant can be made to assume some of the A characters by rapid successive transplanting in bouillon.
4. The B mutant can usually be found in older transplants of A.
5. The two types may be distinguished by certain cultural characters, by slightly different agglutination reactions, and by different degrees of virulence.

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AN INFECTIOUS OPHTHALMIA OF CATTLE.

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PLATE 12.

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During the months of September and October, 1922, there occurred among cattle recently introduced into a dairy herd a considerable number of cases of ophthalmia. Inasmuch as cases of pneumonia associated with *Bacillus bovisepticus* appeared among the animals at the same time the two diseases were confused. At first it was thought that the ophthalmia might be a local manifestation of the infection with *Bacillus bovisepticus*. Among the first cases examined were several which showed unmistakable evidence of respiratory diseases as well as acute ophthalmia. The results of bacteriological examination soon eliminated *Bacillus bovisepticus* as the possible etiological agent.

The disease was introduced among cows recently purchased from a dealer in Ohio. Several were suffering from ophthalmia when received. These animals were housed in a barn with two other lots of newly purchased cows. Eleven of these subsequently became infected. Six other cows in an adjoining barn also developed the same disease. In all, it was possible to isolate a characteristic organism from twenty-four cases.

Clinical Characters.

The infection was characterized by its sudden onset. There was marked photophobia and impairment of vision. Lacrimation was excessive and accompanied by considerable thick, yellow discharge which gathered at the inner canthus. The exudate often washed downwards soiling the hairs of the face (Fig. 1). The eyelids were thickened. Palpation of the lids caused considerable discomfort.

The membrana nictitans was often deeply reddened and swollen. The conjunctiva was bright red in color. The vessels of the eyeball were injected. Fine strings of the yellowish white exudate were often observed on the eyeball or conjunctiva. In a few instances shallow, irregular ulcers were encountered on the cornea. In three cases there was extensive corneal opacity. Here interference with vision was severe. It was feared that the sight would be lost, but the opacities became smaller and finally disappeared. In certain cows but one eye was affected, in others both eyes were involved.

Bacteriology.

Fresh exudate was obtained from the inner canthus and beneath the lids on sterile cotton swabs. Films were prepared on sterile cover-slips. The remaining material was suspended in salt solution and blood agar plates prepared at once. At first the swabs were smeared directly over slants of various media but the usual presence of spreading colonies of *Bacillus subtilis*, molds, etc., frequently made identification difficult. On the whole, when the organisms were numerous the plates from the salt solution suspensions were more satisfactory. Films of the exudate when stained with methylene blue or other aqueous stains usually revealed long strands of mucus. In certain cases the exudate was apparently composed only of mucus (Fig. 2) with a few epithelial cells. In others the exudate contained leucocytes in large numbers (Fig. 3). Exudates when stained by Weigert's method often showed the presence of fibrin in small amounts. The organisms were usually abundant. The bacterium most frequently observed was a short rod with rounded ends. It usually occurred in pairs. Morphologically it appeared indistinguishable from published figures of the Morax[1]-Axenfeld[2] diplobacillus of human pink-eye. At first it was regarded as Gram-positive. After staining with Stirling's gentian violet and fixing with Lugol's solution it resisted decolorization with alcohol to a considerable degree but eventually became decolorized.

[1] Morax, V., *Ann. Inst. Pasteur*, 1896, x, 337.

[2] Axenfeld, T., *Centr. Bakt.*, *1te Abt.*, 1897, xxi, 1.

Morphological and Cultural Characters of the Diplobacillus.

Morphology.—The individual elements are short, plump bacilli with rounded ends. The length is fairly uniform in 24 or 48 hour cultures, varying from 1.5 to 2 microns, and the width is 0.5 micron. Longer forms are not infrequent in the exudate. The bacilli usually occur in pairs and short chains both in the exudate and in culture media. Chains of four to eight are common in bouillon. It is non-motile and does not form spores. Well developed capsules may be demonstrated in suitable preparations. The organism stains well by ordinary methods and it is Gram-negative. Growth is better on media containing blood or serum.

Colonies.—In agar plates containing 8 per cent of defibrinated horse blood the colonies are characteristic. After 24 hours the surface colonies are round, translucent, and grayish white. They are surrounded by a narrow, clear zone of hemolysis. The deep colonies are at this time barely perceptible to the naked eye. In sharp contrast is their clear hemolytic zone, usually 1.5 mm. in diameter. After 48 hours the surface colonies are more flattened in appearance and may reach a diameter of 3.5 to 4 mm. (Fig. 4). The deep colonies are ovoid and biconvex in shape. The hemolytic area has increased in diameter to 2.5 to 3 mm.

Blood Agar Slants.—After 24 hours at 38°C. there is a heavy, viscid, grayish white growth which does not tend to spread on longer incubation. The blood in the condensation water is hemolyzed.

Plain and Serum Agar Slants.—These reveal a similar growth but more restricted than that on blood agar.

Coagulated Serum.—On the surface there appear after 18 hours tiny pits which gradually enlarge as incubation continues into saucer-shaped centers of liquefaction. Later the centers of liquefaction coalesce and after 10 days about two-thirds of the medium is liquefied.

Agar and Serum Agar Stab.—The growth is confined to the surface or 1 or 2 mm. along the needle track.

Gelatin.—At 22°C., growth is slow. At first the surface is pitted like the coagulated serum. Within 7 days there is distinct liquefaction which progresses very slowly.

Plain Bouillon.—Growth takes place slowly. There is a slight turbidity with considerable sediment. On agitation the sediment breaks up into coarse particles.

Dextrose Bouillon in Fermentation Tubes.—The growth is confined to the bulb and resembles that in plain bouillon. There is no fermentation. The medium tends to become more alkaline.

Litmus Milk.—The medium becomes more alkaline. After 10 days three distinct zones are visible: an upper, liquid zone of a deep blue color, an intermediate zone lighter in color and containing soft flocculi of casein, and at the bottom of the tube a still lighter portion of coagulated casein.

No growth has been obtained on potato. Acid production has not been noted in any of the carbohydrate media usually employed. Indole is not found in sugar-free broth after 14 days incubation. A temperature of 58–59°C. for 5 minutes kills the organism. When the growth from 24 hour blood agar slants is smeared on sterile cover-slips and dried at room temperature the organism remains viable for 3 days. After this time growth can no longer be obtained.

Pathogenicity for Small Animals.

White mice and rabbits are refractory to subcutaneous or intraperitoneal injection of suspensions of exudate made from the eyes of cattle. In three instances a little of the suspension was dropped into the eyes of normal rabbits. The animals failed to react. Guinea pigs and rabbits injected intraperitoneally with heavy suspensions from freshly isolated cultures remained well. Heavy suspensions of cultures dropped into the eyes of rabbits and guinea pigs failed to produce noticeable effects.

EXPERIMENTAL.

Although the evidence pointed to the diplobacillus as the etiological agent there still existed some doubt as to the possible existence of some underlying agent. It was decided to inoculate fresh suspensions of exudate obtained from acute cases with the hope that if other organisms were responsible they might be detected early in the course of the infection.

With this in view a young calf was first inoculated by dropping a little of the fresh suspension on the eyeball and retaining it beneath the lower lid. The animal developed a mild inflammation of the eyes 2 days later, from which the diplobacilli were recovered. On the whole, the disease produced was so mild that it might have been due to mechanical causes.

A 6 months old bull was subsequently inoculated in the right eye with 0.5 cc. of a suspension of the exudate from two cases. The following protocol gives the results in some detail.

Bull Calf 898.—On Sept. 16, 1922, 0.5 cc. of a salt solution suspension of exudate from two acute cases was dropped on the eyeball and retained for a few seconds beneath the lower lid of the right eye. Sept. 17 to 19. The eye appeared normal. On Sept. 20, a ramiform congestion of the vessels of the eyeball appeared. The membrana nictitans was swollen and of a bright pink color. The conjunctiva was deeply reddened. The eyes were sensitive to light. There was considerable thick, yellowish white discharge at the inner canthus. This on microscopic examination was mucopurulent. Diplobacilli were present in considerable numbers. Plate cultures revealed a few colonies of streptococci and many colonies of diplobacilli. The discharge continued for 3 or 4 days and gradually became less. The diplobacilli were observed in the films and were cultivated in plate cultures. On Sept. 28, the left eye became involved. A large amount of yellowish white exudate was observed at the inner canthus. The membrana nictitans was swollen and red. Microscopic examination of films from the exudate revealed strings of mucus, leucocytes, and a moderate number of diplobacilli. Over 80 per cent of the colonies which developed in the plate cultures were of the characteristic hemolytic type. The inflammation in the other eye was more pronounced at this time. The reaction gradually subsided and within 10 days disappeared. On Oct. 20, the eyes appeared normal, except for a trace of discharge at the inner corner of the right. Films prepared from both eyes still showed diplobacilli. The organisms were numerous and made up 95 per cent of the colonies developing in the plate cultures. Treatments three times a day with a 1:40 solution of zinc sulfate were begun on Oct. 23. A small quantity of the solution was dropped on the eyeball and retained for a brief period beneath the lids. On Oct. 26, the organisms could not be found in films. Plate cultures were also negative. The treatment was continued until Oct. 28. On this day material was again examined with negative results.

The preceding experiment strongly indicated that the diplobacilli were responsible for the disease. Streptococci in small numbers were also present but the diplobacilli made up from 70 to 95 per cent of the organisms present. Their persistence after the acute symptoms had subsided was significant. To establish definitely the etiological

relationship of this diplobacillus pure cultures were dropped in the eyes of two cattle. A detailed report of one case is given.

Cow 897.—On Oct. 10, a Holstein cow that had been under observation for several months was isolated in a tightly screened unit. On this day one-half the growth from pure cultures isolated from two spontaneous cases were each suspended in 2 cc. of sterile bouillon. 6 drops of these suspensions were permitted to run over the eyeballs. The right eye received culture from Cow 4301, the left the suspension from Culture 4316. On Oct. 11, lacrimation was pronounced. On Oct. 12, the eyes appeared normal, except for barely discernible swelling about the lids at the median borders. On Oct. 13, the lids of the right eye were appreciably thickened. The conjunctiva was reddened. The vessels of the eyeball were injected. A small amount of yellowish white, thick discharge was present at the inner canthus. Shreds of exudate were observed on the surface of the eyeball. The condition of the left eye was about the same, but the discharge was more copious. Microscopic examination of exudate from the right eye revealed desquamated epithelial cells, mucus, and large numbers of diplobacilli. Material from the left eye showed polymorphonuclear leucocytes in addition to epithelial cells, mucus, and diplobacilli (Fig. 5). The plate cultures made from salt solution suspensions of exudate developed the hemolytic colonies in practically pure cultures. Observations were made daily. The ophthalmia became more pronounced during the next few days. On Oct. 15, swelling of the lids was pronounced. It extended for a distance of 5 cm. beyond the borders of the lids. About the inner corners of the eye the characteristic yellowish white exudate was observed. Palpation caused considerable discomfort. Congestion of the conjunctiva was pronounced. On Oct. 19, the manifestations of inflammation were sevêre. On Oct. 23, edema of the lids and congestion of the conjunctiva were accompanied by a mucopurulent exudate. The diplobacillus was present in the films and plate cultures in enormous numbers. Treatment with a 1:40 solution of zinc sulfate was started on Oct. 23. The inflammation subsided rapidly. After 3 days it was no longer possible to obtain the organisms in plate cultures. A few diplobacilli could still be found in the films. Treatment was continued until Oct. 28, when all symptoms had disappeared and diplobacilli did not develop in the plate cultures.

Cow 983.—The previous experiment was repeated. On Nov. 1, a bacteriological examination of the eyes failed to show the presence of the diplobacilli. Material obtained from beneath the membrana nictitans revealed only epithelial cells. On Nov. 1, material for inoculation was prepared by suspending three loopfuls of growth from the surface of a 24 hour agar culture in 2 cc. of bouillon. 3 drops of this suspension were instilled into the right eye. 2 days later the eye was visibly inflamed. The condition became more aggravated. 5 days after the inoculation the reddening was pronounced. The membrana nictitans was almost twice its normal size, being reddened and edematous. The lower lid was thickened. Congestion of the conjunctiva was pronounced. The characteristic yellowish white

exudate was observed. Diplobacilli were present in the smears in enormous numbers. During the observation the left eye remained normal. The diplobacilli were first observed in the plate cultures and films prepared from the membrana nictitans of the left eye 10 days after inoculation. The inflammation gradually subsided in the right eye and on Nov. 12 abnormalities could no longer be detected. On Nov. 17, the organisms were still present in material obtained from both nictitating membranes.

On the whole, the disease produced experimentally was milder than that observed in the herd. The fact that the diplobacilli constantly associated with the spontaneous disease are fully capable of reproducing the disease when introduced into the eyes of normal cattle is established by our experiments. Probably the severity of the disease may be influenced by initial injury. The organism is, however, capable of attacking the normal membranes. In our experimental inoculations precautions were always taken against injurying either the eye or the lids. It seems possible to account for the comparative mildness of the inflammations by the conditions under which the cattle were kept. The units were free from draughts. The temperature was fairly uniform and the atmosphere contained very little dust. In addition, flies were not numerous. On the other hand, the spontaneous cases varied considerably in their intensity. The incubation period was relatively a short one. It varied from 2 to 4 days in the experimental cases.

Of considerable significance is the persistence of the etiological agent in two of the three experimental cases after the disease had apparently subsided. Additional evidence was obtained when ten spontaneous cases were reexamined 3 and 4 months after the original observations. In a few cases a little discharge still persisted, but in nine cows a clinical diagnosis of ophthalmia was not justified. There was a pronounced opacity of the cornea in the other animal. From material obtained from the membrana nictitans of five cases diplobacilli were observed in the films and cultivated in plate or coagulated serum cultures.

DISCUSSION.

From the lack of literature it would seem that contagious ophthalmia is relatively rare in cattle. Practitioners in this district, however, inform us that during the early fall months eye diseases are prevalent

in certain localities. Whether these outbreaks are due to infection with the diplobacilli is not known. Allen[3] called attention to a disease of the eyes of cattle in the Province of Quebec. The symptoms manifested differed in certain respects from those observed by us. He called particular attention to a smoky or hazy appearance of the cornea with marked lacrimation and photophobia. The development of protuberances in the membrana nictitans was also recorded. Short, thick diplobacilli were encountered in small numbers in stained smears of the watery exudate. Attempts to cultivate these organisms on ordinary media failed. In one instance a culture was obtained in a serum medium. Attempts to reproduce the disease in cattle by instillation of this culture into the eyes failed.

In a translation by Kappeyney and Ward[4] of an article by Poels[5] an infectious keratitis of cattle is described. From bits of incised cornea Poels cultivated *Bacillus pyogenes*. Instillation of the culture beneath the lids produced no apparent ill effects. Injection of *Bacillus pyogenes* beneath the epithelial layer of the cornea resulted in infection.

In morphology and the ability to liquefy serum the organism isolated by Allen resembles the one obtained by us. There are, however, many clinical differences. In our cases a yellowish white, mucous or mucopurulent discharge was practically a constant accompaniment of the disease. Films from the bulk of the cases revealed the diplobacilli in large numbers. Allen calls attention to the relatively few organisms encountered in a watery exudate. In his cases the principal diagnostic sign was a smoky appearance of the eyeball. This symptom was not observed by us.

The disease did not necessarily attack neighboring cows. The cases occurred irregularly throughout the barn and finally spread to other barns. It seems possible to account for this irregular occurrence by assuming transmission by flies. They were numerous during the outbreak and were frequently observed feeding on the exudate. The disease became quiescent after the inauguration of treatment and the onset of cold weather. It is not impossible that it may

[3] Allen, J. A., *J. Am. Vet. Med. Assn.*, 1918–19, liv, 307.

[4] Kappeyney, J., and Ward, A. R., *J. Am. Vet. Med. Assn.*, 1917, li, 526.

[5] Poels, J., *Tijdschr. veeartsenijk.*, 1911, xxxviii, 758.

reappear in sporadic or epidemic form at any time, since five of the ten cows reexamined after 3 or 4 months were still carrying the diplobacilli. Since the data have been gathered together a typical but mild infection from which diplobacilli were isolated occurred in the latter part of January, 1923.

Of considerable interest is the resemblance of the diplobacillus obtained from the cows to that described by Morax and Axenfeld, Pusey,[6] and others from human conjunctivitis. The morphology appears to be the same. Certain of the growth characters of both organisms are suggestive. Both form the characteristic pits of liquefaction on the surface of coagulated serum. Both are obligatory aerobes. The addition of serum to media enhances growth. Carbohydrate fermentation has not been observed for either organism. Neither is pathogenic for laboratory animals. Of interest is the apparent specificity of zinc sulfate in the treatment of infections with the human and the bovine diplobacilli. The Morax-Axenfeld bacillus is said not to liquefy gelatin. Its failure to grow in milk is a further difference. It may be possible to explain these apparent differences in the light of more recent knowledge concerning the initial hydrogen ion concentration of culture media. The proteolytic activities of the Morax-Axenfeld bacillus in coagulated serum media suggest that under proper conditions of alkalinity digestion of gelatin and casein is possible. Both types must be compared under the same conditions before specific differences can be determined.

SUMMARY.

Twenty-four cases of an acute ophthalmia of cattle have been observed. The infection is characterized by photophobia, severe congestion of the vessels of the eyeball, conjunctivitis, congestion and edema of the membrana nictitans, edema of the eyelids, accompanied by a thick, yellowish white mucus or mucopurulent exudate. In certain cases corneal ulcers and extensive corneal opacities developed. From all cases a characteristic diplobacillus was obtained. The organism was usually observed in the exudate in large numbers. The morphology, the hemolytic properties, and the proteolytic

[6] Pusey, B., *J. Am. Med. Assn.*, 1906, xlvii, 255.

activities readily assist in its identification. Instillation of a few drops of bouillon suspensions of pure cultures beneath the eyelids of normal cattle gave rise to characteristic inflammations. The organism is not pathogenic for laboratory animals.

EXPLANATION OF PLATE 12.

FIG. 1. A spontaneous case. The lids are thickened. The characteristic exudate has accumulated at the inner canthus and has been washed down over the face.

FIG. 2. Exudate from a spontaneous case. The diplobacilli are visible in the mucous exudate. $\times$ 1,000.

FIG. 3. Diplobacilli in the mucopurulent exudate obtained from a spontaneous case. $\times$ 1,000.

FIG. 4. Colonies in a horse blood agar plate culture after 48 hours incubation. The hemolytic areas about both the surface and deep colonies are well defined. Natural size.

FIG. 5. Mucopurulent exudate obtained from Cow 897, an experimental case. $\times$ 1,000.

(Jones and Little: Infectious ophthalmia of cattle.)

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THE SURVIVAL OF BACTERIA IN THE PUPAL AND ADULT STAGES OF FLIES.

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INTRODUCTION.

Before attempting to study the transmission of microörganisms by flies, whether these microörganisms are pathogenic to the flies themselves or to higher animals, it is necessary to obtain some idea of the extent of the normal intestinal flora. It is a well known fact that the maggots of many flies live in the faeces of vertebrates and consequently become infected with countless bacteria. As a matter of fact, the recent work of Guyénot, Loeb, Baumberger, Wollman and others has shown that it is extremely difficult, if not impossible, to rear fly larvae in the absence of bacteria or yeasts, as some essential food requirement (vitamines?) is obtained from these microörganisms. Whether very many pathogenic bacteria can survive the environment encountered in the larval intestine, and later pass on and survive in the pupal and adult stage of flies, has been a much debated question. In the larval fly intestine, organisms pathogenic to higher animals are confronted with two known factors that are inimical to their propagation and survival. They are first subjected to all the various harmful influences produced by all the other species of bacteria, and are also subjected to the action of the host's digestive secretions which are liberating the vitamines, so essential to the life of the larva.

A study of the degree of general bacterial infection of pupae and recently emerged adults would show whether bacteria could survive the conditions encountered in the larval and pupal stages and pass on to the adult intestine, to be disseminated by defaecations and

regurgitations. This question of the survival of bacteria from larva to adult has nothing to do with the well known fact that many adult flies habitually ingest and spread countless bacteria, many of which are pathogenic.

In 1909, Faichnie found that six out of thirteen house flies bred from a typhoid stool contained *B. typhosus* in their intestines. Similarly, four out of eleven flies fed from a paratyphoid stool harbored *B. paratyphosus* A.

Bacot (1911) performed some experiments with house flies bred in material contaminated with *B. pyocyaneus*. This work proved that the microörganism used was ingested by the *M. domestica* larvae, survived during the complex changes accompanying metamorphosis and continued its existence in the intestine of the adult.

Ledingham, in 1911, after feeding the larvae of house flies on material contaminated with *B. typhosus* isolated this microörganism later from the interior of the pupae. He concludes, however, that "the typhoid bacillus can only lead a very precarious existence in the interior of the larvae or pupae which possess, at least in so far as these investigations warrant, a well defined bacterial flora of their own." This latter contention was verified by Nicoll (1911) and in the same year Graham-Smith, in his work with blow fly larvae, found that "none of the non-spore-bearing organisms such as *B. prodigiosus*, *B. enteritidis* and certain cocci survive sufficiently long to be found in the blow flies which emerge from infected larvae."

Nicoll (1912) infected the larvae of *Sarcophagula* and *Sarcophaga* with *B. typhosus* and found that during development the fly possesses great powers of destroying bacteria. When larvae of *Sarcophagula*, breeding in faeces contaminated with *B. typhosus*, were removed from the infected medium the bacilli rapidly disappeared from the larvae. He further observed that newly emerged adult flies were practically sterile.

Graham-Smith, from the results of his own work in 1909–1913 and from the work of others, finally summarizes the entire situation in 1914 thus: "Though it seems to have been proved that the spores of *B. anthracis* may survive, most observers agree that such non-spore-bearing pathogenic organisms as *B. typhosus*, *B. enteritidis* and *B. dysenteriae* derived from cultures and added to the food of the

larvae are not present in the flies which emerge, except under very special and highly artificial conditions. Most of these observers conclude from their experiments that the possibility of flies becoming infected from the presence of pathogenic organisms in the breeding ground of the larvae may be considered as remote. On the other hand, Faichnie, working with uncultivated *B. typhosus* and *B. paratyphosus* A, stated that he was able to isolate these organisms from the flies which emerged. All the other investigators have failed to take into account the possibility of cultivated bacilli behaving in a different manner to uncultivated bacilli."

"One point, however, has been clearly demonstrated. Both the larva and adult fly have a peculiar flora, consisting so far as is at present known of non-lactose fermenting organisms, adapted to life within their alimentary canals and capable of surviving the pupal stage. These bacilli are of considerable practical importance since they are often present in large numbers and render the search for pathogenic bacilli of the typhoid-enteritidis group one of great labor, since they resemble them in many cultural characters and can only be certainly distinguished from them by means of elaborate cultural and serological tests."

The work on the problem in question has been done heretofore on the non-blood-sucking muscids, such as *M. domestica*, *Lucilia caesar*, *Calliphora erythrocephala* and *Sarcophaga* sp.? No one seems to have attempted an inquiry into the situation as it exists in the biting flies. Since the biting muscids are also important disease transmitters to man and, especially, to our domestic animals, the problem was considered worthy of investigation. *Stomoxys calcitrans* (the biting stable fly) and *Lyperosia irritans* (the cattle horn fly) were the principal subjects used for the present work. The problem was also reinvestigated in the house fly, not for the purpose of verifying the careful and extended researches of others on this form, but for comparative reasons. It was thought that the situation might possibly be slightly different in the region in which this work was done, so it would be unsafe to compare the results obtained with the biting forms with the published data on *M. domestica*.

No work was attempted on the persistence of *pathogenic* bacteria from the larval to the pupal and adult stage. The present study is

simply a general quantitative one and no attempt was made to accurately identify and classify the various species of bacteria encountered. At times it was found necessary to penetrate a little deeper into the characteristics of a number of pure cultures, in order to see whether one or several species existed in a particular experiment. However, as soon as the ordinary morphological, cultural and sugar fermentation tests convinced the writer that he was dealing with separate forms, the diagnostic work was stopped.

As will be seen from the work to be described, an attempt was made to answer the questions: To what extent can bacteria, generally speaking, persist in biting flies from the larval through to the pupal and adult stage? And, is the bacterial flora of the blood-feeding muscids as extensive as that of the non-biting forms?

DESCRIPTION OF THE EXPERIMENTS.

In the first series of experiments, eight recently pupated house flies were removed from horse manure. The pupae were gently scrubbed in tap water until all the grosser dirt was removed from the exterior. They were then rapidly washed in a mixture consisting of one half absolute alcohol and one half 1:1000 corrosive sublimate solution. After this the pupae were transferred to a fresh mixture of alcohol and corrosive sublimate in which they remained for five minutes. While in this solution, the pupae were agitated at intervals by shaking the tubes. This disposed of air bubbles adhering to the surface of the pupae and caused them to sink to the bottom. At the conclusion of the five minutes, the pupae were washed once in sterile distilled water and then, following the method of Bacot and Ledingham (1911), twice in nutrient broth. Two separate nutrient broth tubes were used for each pupa, making sixteen tubes in all. After the removal of the pupae, these tubes were incubated for 48 hours at 37°C. in order to see whether the sterilization of the exterior of the pupae had been effective. No growths were obtained in any of the broth tubes.

At the conclusion of the washings in bouillon each pupa was transferred to a separate nutrient agar slant. Half the number were crushed and streaked over the surface while the other half were left entire, simply resting on the slants or in the water of condensa-

tion. These tubes were kept at room temperature. The water of condensation in which pupa No. 1 rested became slightly turbid in 24 hours. In 48 hours the growth had spread over most of the slant. The bacterial contamination in this case, as in all "not crushed" pupae, came from the spiracular openings. Pupa No. 2 rested on the slant. No growth was visible until 72 hours when a heavy luxuriance began to appear around the vicinity of the spiracular openings. Similarly, in pupae Nos. 3 and 4 nothing was visible until 48 hours, when growths appeared around the respiratory openings. Bacot (1911) in some of his experiments also noticed this growth of bacteria from the spiracles of pupae that had been thoroughly sterilized on the exterior. It seems, therefore, that during pupation bacteria become lodged in the upper air passages. The four pupae that were crushed and streaked over the slants yielded a heavy growth in 24 hours.

Another set of experiments with five house fly pupae was then performed. The preliminary treatment of the pupae was identical to that used in the preceding set, except that the subjects were left in the sterilizing agent for ten minutes. After the two washings in bouillon, the pupae were mounted with one end up in soft sterile paraffin. The exposed end was cut off with sterile scissors, and a sterile platinum rod was inserted with which the contents were stirred after the manner of a soft boiled egg with a spoon. The five pupae were so treated, and after each treatment the rod was streaked three times on a separate nutrient agar plate. The five plates were incubated for 24 hours at 37°C.

The results obtained after the incubation may be summarized thus: Although the first and second sets of bouillon tubes used in washing the pupae were incubated for 48 hours, no growths were obtained, showing that the sterilization of the exterior was complete. After 24 hours incubation, the first plate representing pupa No. 1 yielded a heavy diffuse growth covering the entire surface of the medium. The second plate, representing pupa No. 2, gave a moderate growth along the first streak. Plates 3, 4, and 5 yielded heavy diffuse growths.

Series III represents a quantitative study of the bacteria in five house fly pupae. These results can be compared later with those

obtained for *Stomoxys* and *Lyperosia* pupae, since all the quantitative work was executed in an identical manner. After the preliminary treatment and the sojourn in the sterilizing agent for ten minutes, followed by the two washings in bouillon, a small sterile platinum loop[1] was inserted into the pupa. The contents were well mixed and then one loopful was removed and deposited into a tube containing 12 c.c. of physiological salt solution. This tube was well rotated, after which two loopfuls from this tube were taken and deposited into a cooled, melted nutrient agar tube containing 12 c.c. of medium. After rotation three loopfuls were removed and deposited into a second nutrient agar tube (12 c.c. of medium). After the second agar tube had been rotated, both media tubes were plated. The plates were incubated for 72 hours at 37°C. and colony counts were made at the end of 48 and 72 hours.

The results obtained were as follows: After the customary incubation for 48 hours none of the two sets of bouillon tubes used in washing the pupae yielded growths. On the first dilution plate pupa No. 1 gave two colonies in 48 hours and 18 in 72 hours. Pupa No. 2 gave 4 colonies in 48 hours and 5 in 72 hours. Pupa No. 3 gave 2 colonies in 48 hours. Pupa No. 4 gave 5 colonies in 48 hours and 8 in 72 hours. Pupa No. 5 gave 2 colonies in 48 hours with no increase in 72 hours. No colonies were obtained on any of the second dilution plates.

Series IV represents some work done with recently emerged, reared house flies. These experiments covered a wide range of time until concluded, because it was important to take house flies as soon as they emerged, before feeding, in order to determine how many bacteria passed through the pupal stage and survived in the adult intestine. Bacteria ingested during adult life must be excluded in so far as possible. Therefore, one must catch flies in the act of emerging and take them as soon as they are free from the pupal envelope and before they have had an opportunity to feed. The flies were usually helped out of the encumbrance and immediately placed in sterile phials in which they were permitted to harden before being used.

[1] The same small, standard loop was used throughout the work.

The exterior of adult flies is difficult to sterilize owing to the fact that they are covered with so many hairs, setae and bristles. However, the writer has found the following procedure fairly satisfactory. The flies were first etherized, after which the legs, wings, and most of the bristles and setae were removed with fine scissors. The flies were then washed twice in sterile water, after which they were washed in the corrosive-sublimate-alcohol mixture. They were then placed into fresh sterilizing mixture and left for from 5 to 10 minutes. Then followed the washing in sterile distilled water and the customary two washings in nutrient bouillon. In series IV, experiment No. 2 was the only one from which one cannot draw any conclusions as to whether the bacteria counted on the plates originated from the exterior or interior of the fly, for a surface growth was obtained in the second bouillon tube in 48 hours. After the preliminary treatment, each fly was handled in the following way: Two standard drops of sterile physiological salt solution were deposited on a sterile depression slide. Then on another section of the slide the entire intestinal tract of the fly was removed and placed in the solution. Here the intestine was finely triturated with teasing needles. In all the experiments with adult flies in which this procedure was used, uniformity was the aim. Of course, it would be absurd to attempt to move the needles in scissor fashion the same number of times for each fly intestine. Standardization can be much more accurately obtained through the eye of the observer. The intestines in each case were triturated until approximately the same particle size and color consistency of the material was obtained. A comparison was always made with a specimen kept near at hand, over the same color background and called the standard. With practice this procedure was found to be almost as accurate as the colorimetric readings used so much in chemical work. From the triturated material, one standard loopful was deposited into 12 c.c. of physiological salt solution and the further two, three dilution with nutrient agar practiced as was the case with the experiments on the pupae. At the same time one loopful of the material was also deposited on a slide; a film was drawn out, fixed and stained with Loeffler's methylene blue. The plates were incubated for 48 hours at 37°C. The growths on the five first dilution plates were too thick and confluent for counting. On the

second dilution plates, fly No. 1 gave 30 colonies; fly No. 2 gave 176; fly No. 3 gave 36; fly No. 4 gave 164, and fly No. 5 gave 50. The stained films prepared from the intestines showed numerous bacteria.

Series V was performed with five wild house flies. The work was executed in precisely the same manner as in the previous case. No growths were obtained in the two sets of bouillon washings. After 48 hours incubation the first and second dilution plates, representing fly No. 1, gave colony counts 9 and 0 respectively. For fly No. 2, the counts were 6232 and 38; for fly No. 3, 548 and 4; for fly No. 4, 573 and 4; and for fly No. 5, 72 and 2. The stained films prepared from the intestines showed numerous bacteria.

Series VI represents work done with five pupae of *Stomoxys calcitrans* and was executed in the same manner as the experiments with house fly pupae given in Series III, with the exception that the pupae were here kept in the sterilizing agent for varying times (5 to 36 minutes). No growths were obtained in the bouillon tubes. On the plates, pupa No. 1 yielded no colonies in 48 nor in 72 hours. Pupa No. 2 gave 4 colonies on the first plate and none on the second. There was no increase in 72 hours. Pupa No. 3 gave no colonies in 48 hours nor in 72 hours. The same holds for pupa No. 4. In 48 hours pupa No. 5 gave 176 colonies on the first dilution plate and 1 colony on the second. In 72 hours there was an increase to 181 on the first plate, but none on the second.

Series VII, VIII, and IX were performed with adult *Stomoxys*. The procedure was identical with that used in the experiments with adult house flies. In Series VII, five reared and recently emerged adult *Stomoxys* were used. In the experiment with fly No. 1, no growth was obtained in either washing in bouillon. With fly No. 2, however, a pure culture of a bacillus was obtained in the second bouillon tube. Fly No. 3 yielded a pure culture of apparently the same bacillus in both bouillon tubes. Flies 4 and 5 yielded no growth in either of the bouillon tubes. The colony counts in 48 hours were as follows: Fly No. 1 gave 234 colonies on the first dilution plate and 1 colony on the second. Fly No. 2 gave 400 and 6; fly No. 3 gave 547 and 0; fly No. 4 gave 243 and 6; fly No. 5 yielded no colonies on either plate. The stained films from the intestines demonstrated no bacteria for the first two and the fifth flies. The films from flies Nos. 3 and 4, however, demonstrated bacteria.

Series VIII was performed with five wild adult *Stomoxys.* No growths were obtained in any of the first bouillon tubes used in washing the insects. In the second set of tubes, however, a pure culture of a bacillus was obtained in experiments Nos. 1 and 5. The tubes for experiments Nos. 2, 3, and 4 remained sterile. The colony counts in 48 hours were: Fly No. 1 gave 277 colonies on the first dilution plate and 2 on the second; fly No. 2 gave 1 and 0; fly No. 3, 5 and 0; fly No. 4, 9 and 1; fly No. 5, 5 and 0. The stained intestinal films demonstrated no bacteria.

Series IX comprises another set of experiments performed with five wild adult *Stomoxys.* Fly No. 4 was the only individual that yielded a growth in the bouillon tubes. The colony counts after 48 hours were: Fly No. 1 gave 8 colonies on the first dilution plate and 0 on the second; fly No. 2 gave 11 and 0; fly No. 3, 0 and 0; fly No. 4, 63 and 0; fly No. 5, 1 and 0. The stained intestinal films demonstrated no bacteria.

Series X, XI, and XII were performed with the pupae of *Lyperosia irritans.* The experiments in Series X were executed in the same manner as those in Series I. The results also were practically identical, with the exception that a decided quantitative difference between the number of bacteria in house fly and *Lyperosia* pupae was noticed. When the pupae of *Lyperosia* were crushed on the slants the resulting growth was not nearly as heavy as was the case with the house fly pupae. A microscopical and cultural study seemed to show that only one or two species of bacteria were involved, whereas in the similar house fly experiments one often obtained five or six and even more forms. The *Lyperosia* pupae that were not crushed did not yield any growth for 96 hours, when a luxuriance began to appear around the spiracles. This result conforms with that observed for house fly pupae and again shows that during pupation bacteria also become lodged in the outer respiratory tract. These bacteria in time wander or grow out if the pupae are left in the media long enough.

Series XI was executed in a manner identical with Series II. The bouillon tubes yielded no growth. After 48 hours incubation the streaks on the agar plates gave the following results: No growth was obtained on the two plates representing pupae Nos. 1 and 4. The plate

representing pupa No. 2 yielded a weak diffuse growth. The plate representing pupa No. 3 gave a weak growth along the first streak only. The plate representing pupa No. 5 gave a weak growth along the first and second streaks and no growth along the third.

Series XII was performed with five *Lyperosia* pupae and the method of procedure was identical to the similar experiments represented by Series III (house fly pupae) and Series VI (*Stomoxys* pupae). After the sterilization of the exteriors and the subsequent treatment with sterile water, the pupae were washed twice in bouillon as usual. No growths were obtained in any of these bouillon washings. The colony counts on the plates after 72 hours incubation gave: For pupa No. 1, 1 colony on the first dilution plate and 0 on the second. For pupa No. 2 the result was 0 and 0; for No. 3, 2 and 0; for No. 4, 0 and 0; for No. 5, 1 and 0.

Series XIII and XIV were performed with the adults of *Lyperosia irritans*. These experiments followed the procedure adopted for the adults of *M. domestica* and *Stomoxys calcitrans*. In series XIII, five reared and newly emerged *Lyperosia* adults were used. The bouillon washings did not show growth after incubation for 48 hours. The plates prepared from the triturated intestine gave the following result after incubation for 48 hours: No colonies were obtained on any of the plates excepting Plate 1 representing fly No. 2, where a count of 2 was made. The stained films from the intestine did not demonstrate any bacteria.

Series XIV was performed with five wild *Lyperosia* adults. The results were almost identical to those of the preceding set. Fly No. 1 yielded 9 and 0 colonies on plates 1 and 2 respectively. Fly No. 2 did not give any colonies; fly No. 3 gave 2 and 0 on plates 1 and 2 respectively; fly No. 4 gave 1 and 0, and fly No. 5 nothing. No bacteria were found on any of the stained films.

DISCUSSION AND CONCLUSIONS.

Consideration of the experiments, as described, leads the writer to make the following remarks:

House fly larvae, on pupating, enclose a considerable number of bacteria within them. These bacteria persist through metamorphosis and "pass on" to the adult intestine as was shown in the experiments

with recently emerged flies. Bacteria are also found in the upper regions of the air passages of pupae; but what later fate awaits them remains unknown. The adult *M. domestica* intestine therefore, whether of recently emerged flies that have not fed or of wild adults of unknown age, is a veritable reservoir for bacteria.

Stomoxys larvae, on pupating, also enclose bacteria within them, but in general a much smaller number per individual than is the case with the house fly. This can be gathered by a comparison of the house fly data with those of *Stomoxys* pupae (Series VI) and recently emerged flies that have not fed (Series VII). Wild adult *Stomoxys* of unknown age also harbor bacteria in their intestinal tract, but the number is very small. It must be remembered that the colony counts made in those experiments in which growth was also obtained in one or both bouillon washings must be discarded. In such experiments the colony count is high, but it means nothing since most of the bacteria may have been derived from the exterior of the flies. In other words, in the dissection of the intestines out of the adult flies, contamination with the exterior (which in these cases was not sterile) is unavoidable.

In Series VII flies Nos. 1 and 4, although their exteriors were properly sterilized, gave a high colony count on the first plates. In comparison with the wild cases that can be legitimately used (Series VIII, Nos. 2, 3, 4 and Series IX, Nos. 1, 2, 3, and 5) this seems absurd. Wild flies that have lived longer should harbor more bacteria. It may be that the intestine of the adult *Stomoxys* inhibits the growth of bacteria that pass through from the larva and thus gradually, as the adult ages, many forms are eliminated. It must also be remembered that new bacteria are not included in the menu of the daily diet of *Stomoxys* as is the case with the omnivorous house fly, for the former feeds on sterile vertebrate blood. This food, taken into the fly intestine, may also have an inhibiting effect on many of the bacteria previously derived from the manure during larval life.

The great differences observed between pupal colony counts and counts from the intestine of recently emerged adult house flies and *Stomoxys* may be explained by the fact that the entire intestine was used in the case of the adult, whereas it was impossible to do more

than dip the platinum loop into the agitated contents of the pupa. Thus the loop taken from the pupa represents a sample not from the intestine alone but from the entire semi-liquid contents.[1]

Horn fly pupae contain very few bacteria, as was seen in the description of Series X–XII. The bacterial count of the intestinal material of recently emerged horn flies is extremely low (Series XIII) and the same thing is true for wild horn flies of unknown age (Series XIV). The microscopic examination of intestinal material from adult *Lyperosia*, reared or wild, is very striking in the almost complete absence of bacteria. *Lyperosia* larvae live in cow dung and ingest quantities of bacteria, as can be demonstrated by a cultural or microscopic study of larval intestines. How can one, therefore, explain the low pupal flora and practical adult intestinal sterility? This is difficult until investigated, but probably the bacteria are inhibited and destroyed as the animal transforms and assumes adult life.

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THE DETERMINATION OF FIBRINOGEN BY PRECIPITATION WITH SODIUM SULFATE COMPARED WITH THE PRECIPITATION OF FIBRIN BY THE ADDITION OF CALCIUM CHLORIDE.

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Recently we have had occasion to make a considerable number of determinations of fibrin by the method of Cullen and Van Slyke, which we have modified slightly (1). Simultaneous determinations were made by precipitating fibrinogen with 10.6 per cent sodium sulfate at 37°C., and some determinations by the methods of Gram (2) and of Foster and Whipple (3). The fundamental procedures of various recent methods for the determination of fibrin and fibrinogen are as follows:

(*a*). Cullen and Van Slyke (4). The fibrinogen of oxalated or citrated plasma is permitted to clot by the addition of calcium chloride. The nitrogen content of the fibrin is determined after suitable washing.

(*b*). Howe (*a*). This procedure is identical in part with the above, but modified by the use of smaller quantities of plasma, a greater dilution during coagulation, and the analysis of an aliquot of the filtrate. The two methods give essentially similar results.

(*c*). Gram. Procedure similar to that of Cullen and Van Slyke so far as the coagulation is concerned, but differs in that the fibrin is washed with water, dehydrated with alcohol and ether, and weighed directly.

(*d*). Foster and Whipple. Very similar to the procedure of Gram, but differs in that the fibrin is not dehydrated with alcohol and ether but is dried at 110°C., weighed, ashed, again weighed, and the fibrin calculated by difference.

(*e*). Wu (5). Identical with the procedure of Cullen and Van Slyke, but modified in that the fibrin is analyzed by a colorimetric procedure. No attempt has been made to use this method; for some purposes it should be very valuable.

(*f*). Howe (*b*). Precipitation with 10.6 per cent sodium sulfate at 37°C. This procedure has not been described, but data obtained with it have been published

(6). It is an extension of the method proposed for the determination of the proteins of blood (1). It is not necessary to use sodium sulfate. The following molecular concentrations (grams of salt in a liter of water) of other salts will give essentially the same results—1.0 molar lithium sulfate; 1.25 molar magnesium sulfate; 0.9 molar, with regard to the phosphate radicle, of a mixture of mono- and disodium phosphates in the proportion of 1:2; 1.125 molar solution of a similar mixture of potassium phosphates; or a 3.75 molar solution of sodium chloride. The procedure for sodium sulfate is as follows: Pipette 0.5 cc. of oxalated or citrated plasma into a suitable test-tube, add 15 cc. of 11 per cent sodium sulfate solution[1] at 37°C. After the precipitate has settled,[2] or better after 3 hours, filter on a dry filter, and take 5 cc. of the filtrate for analysis according to any suitable micro nitrogen method.

In some of our work determinations have been made by a number of different procedures, but the data presented concern chiefly a comparison of procedures (*b*) and (*f*). Following the appearance of the procedures of Gram and of Foster and Whipple, determinations were made by these methods, particularly by a combination of the two methods in which the fibrin was removed after coagulation according to Foster and Whipple and dehydrated according to Gram. The data presented are of interest in that essentially the same results were obtained by methods which involve apparently different types of reaction for precipitation.

EXPERIMENTAL.

A series of 80 determinations on different samples of plasma according to procedure (*f*) was compared with a similar series according to procedure (*b*). Data according to (*b*) were taken as the basis of comparison. In the course of the analytical work when the results by the two methods did not agree the determination by procedure (*b*) was repeated. We are, then, comparing in most cases verified values by method (*b*) with unverified values by method (*f*). The total protein nitrogen content of the plasmas ranged from 0.60 to 1.39 gm. of nitrogen per 100 cc. of plasma, and the fibrin values from 0.028 to 0.233 gm. of nitrogen per 100 cc. of plasma. The high total

[1] The volume of 11 per cent sodium sulfate used when added to the 0.5 cc. of plasma gives a final concentration of approximately 10.6 per cent of sodium sulfate, or a 0.75 volume-molecular solution.

[2] The precipitate as it first forms has the appearance of fine needle-like crystals or of finely divided metal in gasoline. This form soon changes into a flocculent precipitate. This phenomenon has not been observed with any other protein fraction, where the precipitation of the first fraction occurs, *e.g.* euglobulin in serum or pseudoglobulin II in new-born calf serum.

nitrogen and high fibrin values did not necessarily coincide. Furthermore, we are comparing results for fibrin with those for fibrinogen. Data on all samples analyzed as indicated are included, there was no selection of material.

An analysis of the data shows the average variation of results by the sodium sulfate precipitation from those of the calcium chloride coagulation to be 0.004 gm. of nitrogen per 100 cc. of plasma, in favor of the sodium sulfate precipitation. If we eliminate the determinations, fifteen, which showed a variation greater than 0.04 gm. of nitrogen per 100 cc. of plasma, the average variation is 0.0004 gm. of nitrogen. The distribution of the plus and minus differences is nearly even, with a tendency in favor of the sodium sulfate precipitation. In forty-one cases the quantity of nitrogen precipitated by sodium sulfate was greater than by calcium chloride, and in thirty-one cases it was the reverse, while there were eight cases in which the amount of protein nitrogen was the same. From the data we have concluded that the results obtained by the determination of fibrinogen by precipitation with 10.6 per cent sodium sulfate at 37°C. are comparable with those obtained by the coagulation of fibrinogen with calcium chloride from citrated plasma at room temperature. It is to be remembered that there is a similarity in the two procedures in that in both cases the filtrate was analyzed and the result subtracted from the same value for total nitrogen.

In the experiments on the weight of fibrin obtained from plasma as compared with the determination of nitrogen, it was found by preliminary determinations that the results by the Gram method were the same as those of the Foster-Whipple procedure within the limits of error of the methods. In subsequent work, therefore, certain details from each procedure were used, as follows: 2 cc. of plasma were measured accurately, to this were added 40 cc. of 0.8 per cent NaCl and 2 cc. of 2.5 per cent $CaCl_2$. After the fibrin had formed it was removed according to the procedure of Foster and Whipple. From this point the fibrin was dehydrated with alcohol and ether and weighed according to the technique of Gram. The solution remaining after the removal of the fibrin was filtered on a dry filter and analyzed according to procedure (*b*) above. For comparison with these results the fibrin formed in the determination by procedure

(*b*) from 0.5 cc. of plasma was similarly removed and treated and the solution analyzed.

A series of thirteen plasmas was analyzed in this manner. The total nitrogen varied from 0.804 to 1.445 gm. of nitrogen per 100 cc. of plasma, and the fibrin from 0.047 to 0.155 gm. of nitrogen per 100 cc. of plasma. The averages of the determinations for the 0.5 and 2 cc. samples, expressed with relation to 100 cc. of plasma, were 0.106 and 0.105 gm.; weight of fibrin 0.658 and 0.659 gm. These results give 16.1 and 15.95 per cent, respectively, for the weights of fibrin obtained from 0.5 and 2 cc. samples. The percentage values are given only as an indication that the relation between the nitrogen determined and the weight of the fibrin found is within reasonable limits, for the quantities of plasma used are too small for any other deduction.

DISCUSSION AND CONCLUSIONS.

From the figures presented it appears that any one of the procedures proposed for the determination of fibrin or fibrinogen is satisfactory. The significance of the data is not confined to the relative accuracy of the procedures nor to the development of a new method. The Cullen-Van Slyke method, or our modification of this method, is in many ways preferable to the use of sodium sulfate at 37°C. when it is desired to determine fibrin nitrogen, because it can be carried out at room temperature. If fibrin alone is to be estimated, the combination of the Foster-Whipple and Gram methods is preferable. The fact that 10.6 per cent sodium sulfate, an approximately 0.75 volume-molecular solution, yields essentially the same results as the formation of fibrin from fibrinogen by the addition of calcium chloride to citrated plasma is of importance. This fact brings the determination of fibrinogen into harmony with the other concentrations of sodium sulfate used in estimating proteins (1). Thus it appears that the concentration of sodium sulfate required for the precipitation of the various "protein fractions" of blood after the first fraction is obtained is 0.25 molar. The concentrations of sodium sulfate which yield the various fractions are: fibrinogen, 0.75 molar (10.6 per cent); euglobulin, 1.00 molar (14.2 per cent); pseudoglobulin I, 1.25 molar

(17.7 per cent); and pseudoglobulin II, 1.50 molar (21.5 per cent).[3] The first two fractions give results which agree very closely with those obtained by other procedures in which the precipitating factor is not that of "salting out," as the term is ordinarily used; *i.e.*, fibrin by recalcification and euglobulin by dilution and acidification with carbon dioxide.

The fact that similar results were obtained for fibrin by the two procedures used also indicates that the "overlapping" of precipitation limits or contamination of precipitates by adsorption is approximately the same in either process. This conclusion is reached from a consideration of the different bloods used; it did not matter whether the blood was rich or poor in fibrin or contained none of the next protein fraction, new-born calf, or was rich in euglobulin.

There may be some question as to the propriety of comparing results for the determination of fibrin and those for fibrinogen.[4] The question of the presence of Hammarsten's fibrinoglobulin (8) has not been entirely settled. The analyses of the blood plasma and serum of new-born calves suggest that there is not a protein present which requires a salt concentration for precipitation other than that required for fibrinogen, or if it is present it is not in readily demonstrable amounts. Thus in the plasma a precipitate is completed at 10.6 per cent of sodium sulfate and there is not a further appreciable precipitate until after 17.7 per cent sodium sulfate has been added, and in serum no appreciable precipitate occurs until after 17.7 per cent of the salt is present. In the latter case euglobulin is not present to interfere with the observation by a possible overlapping of precipitation limits. The similarity of the results by the various methods outlined above is suggestive in this connection.

[3] The concentrations for euglobulin and pseudoglobulin I given are slightly different from those first proposed, 13.5 to 14.5 and 17.4 per cent of sodium sulfate, respectively. The concentrations given above were found to give the same results with blood serum and are the optimum concentrations for colostrum (7).

[4] That fibrinogen was present in precipitates with salt solutions was demonstrated by dissolving the precipitate in water and adding serum; fibrin was formed.

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THE RELATIVE PRECIPITATING CAPACITY OF CERTAIN SALTS WHEN APPLIED TO BLOOD SERUM OR PLASMA AND THE INFLUENCE OF THE CATION IN THE PRECIPITATION OF PROTEINS.*

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Evidence has been presented (1) indicating the occurrence of critical zones in the precipitation of the proteins of blood. Upon the addition of increasing amounts of sodium sulfate at these zones there is not a marked increase in the quantity of protein precipitated for a given increase in salt concentration. The zones occur in the neighborhood of the following concentrations of sodium sulfate at 37°C.: 10.6 per cent (2), 14.2 per cent, 17.7 per cent, 21.5 per cent, or expressed in volume-molar concentrations, 0.75, 1.00, 1.25, and 1.50 molar, respectively. Comparison with certain existing procedures for separating proteins has shown that (*a*) the 0.75 molar solution precipitates the same amount of protein from plasma as that obtained by the addition of calcium chloride to oxalated plasma (2); (*b*) the 1.00 molar solution precipitates essentially the same amount of protein as that obtained by dilution and acidification of serum or plasma with CO_2 (1); (*c*) the 1.25 molar solution does not produce an appreciable precipitate in new-born calf blood, but does give a precipitate in adult blood or in the serum of the new-born calf within a few hours after the absorption of the proteins of colostrum or of blood (3); (*d*) the 1.50 molar solution gives results which approximate the results obtained with saturated magnesium sulfate or one-half saturated ammonium sulfate (1). If we accept the quantity of nitrogen precipitated by a given concentration of sodium sulfate

* Reported before the Society for Experimental Biology and Medicine (Howe, P. E., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 91).

as compared with other procedures which are often considered as evidence of protein fractions of blood, then the data obtained with the concentrations of sodium sulfate given are indicative of the completion of precipitation of fibrinogen, euglobulin, pseudoglobulin I, and pseudoglobulin II, respectively, present in blood plasma. From a qualitative point of view the constancy of the quantitative results obtained by the addition of a definite amount of a salt under definite conditions of temperature and dilution does not necessarily indicate a separation of one protein fraction from another, since any concentration of salt will under such conditions give concordant results with the same sample of serum or plasma. It is because of the latter fact that the critical zones are of importance.

There is a regularity in the increase in the amount of sodium sulfate required for the precipitation of each succeeding protein fraction from blood serum or plasma, 0.25 mol, after the first protein fraction (fibrinogen) is precipitated, which appears to have a bearing on the nature of the salting-out process and the separation of proteins by such procedures. If the series of concentrations of sodium sulfate at 37°C. is extended by 0.25 mol beyond 1.50 mols[1] (total globulin) into the region of concentrations of salts required to precipitate albumin, it has been found that with a 1.75 molar solution a relatively small quantity of protein is precipitated, of a magnitude approximately that precipitated between a 1.25 and a 1.50 molar solution. With a 2.00 molar solution of sodium sulfate a larger precipitation occurs and another large precipitation with 2.25 molar solution. A 2.50 molar solution of sodium sulfate precipitates practically all of the protein, and with a 2.75 molar solution the precipitation of protein is complete. There is a possible significance in the fact that the largest albumin fraction in horse serum and egg white occurs between 1.75 and 2.00 mols of sodium sulfate, while in cow serum the largest fraction is between 2.00 and 2.25 mols of sodium sulfate. Horse serum and egg white contain readily crystallizable albumin, while it is difficult to obtain crystalline albumin from cow serum. The serum of the chicken and the pig show a partition of albumin more like that of horse serum, and the serum of the sheep and man like that of the cow.

[1] Unpublished results.

The data with regard to the regularity of increase in the concentration of salt required to precipitate succeeding globulin fractions have been extended to other salts. This has been accomplished by comparing the results obtained with the concentrations of sodium sulfate given above with those of various concentrations of lithium sulfate, magnesium sulfate, potassium sulfate, mixtures of sodium and potassium phosphate, sodium chloride, potassium chloride, and lithium chloride. Calcium chloride and magnesium chloride have been studied with various results. The data obtained have a significance which is independent of whether or not the quantitative data obtained represent certain protein fractions.

EXPERIMENTAL.

Determinations were made of the nitrogen contained in the filtrates obtained from the precipitation of protein fractions from 0.5 cc. of blood serum or plasma by the addition of 15 cc. portions of various concentrations of salt, calculated on the anhydrous basis; a new portion of blood was taken for each determination. The concentrations of salt actually used were higher than those given in the tables by an amount equal to that required to bring the 0.5 cc. of plasma to the same concentration. The general procedure of precipitation and filtration has been previously described (4). All precipitations were made at 37°C. although it has been found in cases where the salt was sufficiently soluble at ordinary temperatures that similar results may be obtained at room temperature. The data were obtained from time to time on various samples of blood as they became accessible in other connections and they represent results on fresh blood. On the other hand, samples of serum which had been kept for 8 to 10 months gave similar values. The results presented represent concentrations of a given salt required to yield the analytical values obtained with sodium sulfate.

In Table I are summarized data with regard to the different salts.

The following facts are evident from the data presented in Table I:

(*a*). The molar concentration of a salt required for the completion of precipitation of the first protein fraction may be different for different salts.

TABLE I.

*Volume-Molar Concentrations of Salt Required for the Equal Precipitation of Certain Protein Fractions from Blood When Compared with the Results Obtained with Sodium Sulfate.**

Salt.	Protein fractions.				Increment of salt.	
	I	II	III	IV	Mols.	Equivalent of base.
Sodium sulfate	0.75	1.00	1.25	1.50	0.25	0.50
Potassium "	0.75					
Lithium "	1.00	1.40	1.80	2.20	0.40	0.80
Ammonium " †	1.25	1.50	1.75	2.00‡	0.25	0.50
Magnesium "	1.25	1.625	2.00	2.375‡	0.375	0.75
Zinc " §	1.25	1.50	1.75	2.00	0.25	0.50
Sodium phosphate mixture	0.90	1.20	1.50	1.80	0.30	0.50
Potassium " "	1.125	1.425	1.725	2.025	0.30	0.50
Sodium chloride	3.75	5.00‡			5 × 0.25	5 × 0.25
Potassium "	3.75					
Lithium " ¶	5.00	6.00	7.00	8.00	5 × 0.20	5 × 0.20

* No data are given for *magnesium* and *calcium chloride*. If the relation between sulfates and chlorides holds for these two salts, it is impossible to have sufficient magnesium chloride in solution to precipitate the first fraction from plasma. The concentration of calcium chloride required for serum is of the same order of magnitude as that for the chlorides studied. A 5 molar solution of calcium chloride when added to cow serum will give a slight precipitate, but will not precipitate new-born calf serum. If the solutions obtained be allowed to stand, ultimately most of the protein will be precipitated in both cases. These precipitates are insoluble upon the addition of water. It is apparent that in the case of calcium chloride there is a secondary action which is not related specifically to Fraction II or III and which is apparently a general protein reaction.

† The values for ammonium sulfate are not so clear-cut as for the other salts. The data were obtained by determining the protein by the procedure of Wu (5). These values agree closely with those which have formerly been used on the basis of percentage saturation. The analytical values on cow serum obtained with ammonium sulfate agree with those obtained with sodium sulfate. On the other hand, when a concentration for Fraction III is added to new-born calf serum a slight precipitate is obtained with ammonium sulfate, but not with any of the other salts used. No precipitation occurs at 1.58 mols of ammonium sulfate. This is the only case, so far found, in which the absence of precipitation with new-born calf serum has not coincided with the agreement of analytical data on

(*b*). There is a constant additional amount of salt required for each succeeding fraction after the precipitation of the first fraction, called the "increment of salt."

(*c*). The increment of salt required for various salts may be different, but is not necessarily so.

(*d*). The increment of base required for each fraction appears to be the same, or a multiple, for different anions; *i.e.*, there appears to be a definite relation between the various series of salts.

The Influence of the Cation in the Precipitation of Protein.—In Table II data are presented showing the effect of varying the proportions of monosodium (or potassium) and disodium (or potassium) phosphate. The proportions of the two phosphates selected were those used by Sörensen in preparing dilute phosphate solutions having different hydrion concentrations. The hydrion concentrations of the solutions used at the given salt concentrations are not those obtained in dilute solution; they did give the correct hydrion concentration when diluted.

It is apparent from the data presented in Table II, which cover only extreme combinations of phosphates, that:

(*a*). Within a limited range, variations in the relative proportions of monosodium (or potassium) and disodium (or potassium) phosphate, equal precipitation of protein occurs when the concentration of base is the same.

(*b*). When the concentration of the cation is constant the concentration of the PO_4 radicle may vary nearly 0.8 mol without appreciably affecting the analytical results. This fact holds for all the globulin fractions.

cow serum. We prefer for the present to assume that there is an anomalous behavior in the case of ammonium sulfate.

‡ The following are the approximate values in terms of a saturated solution for the salts indicated: ammonium sulfate, 2.00 molar = one-half saturated solution; magnesium sulfate, 2.375 molar = saturated solution; sodium chloride, 5.00 molar = saturated solution.

§ Precipitations with zinc sulfate are not so clear-cut as with the other sulfates; *i.e.*, it is much more difficult to obtain consistent determinations.

¶ It is possible to obtain consistent data with lithium chloride only by filtering soon after precipitation, otherwise the precipitate becomes insoluble and additional protein is also precipitated.

(*c*). The increment of base between the various protein fractions is the same as that for the sulfates. The data presented on mixed salts, given below, make it permissible to assume that if potassium sulfate were sufficiently soluble it would give results similar to those obtained for sodium sulfate.

(*d*). The beginning concentration for the precipitation of the first protein fraction, with regard to the PO_4 ion, is not the same for the sodium and potassium phosphates as it is for the sulfates and chlorides.

(*e*). There is a slightly greater precipitation of protein with the higher proportions of acid phosphate, but this is small in comparison with the quantity of protein precipitated. If the proportion of acid

TABLE II.

Precipitation of Protein by Various Mixtures of Potassium Phosphate. Results Are Expressed as Grams of Nitrogen per 100 Cc. of Plasma and Represent the Quantity of Nitrogen Remaining in the Filtrate after Precipitation.

Salt.	Protein fractions.*			
	I	II	III	IV
Sodium sulfate	0.940	0.791	0.621	0.524
$\frac{KH_2PO_4}{K_2HPO_4}$ ratio.				
$\frac{1}{10}$	0.912	0.795	0.625	0.524
$\frac{1}{2}$	0.932	0.791	0.620	0.522
$\frac{8}{1}$	0.930	0.783	0.593	0.520

* Total nitrogen = 0.993 gm.

phosphate be sufficiently increased, a precipitation occurs which is not related to the concentration of base but to the hydrion concentration.

Table III contains data relating to the variation in the concentration of PO_4 ion which exists when the concentration of base is maintained the same.

Precipitation by Mixtures of Salts.—The regular increment required for the precipitation of the various protein fractions after the concentration for the first fraction has been determined offers an opportunity for comparing mixtures of salts. Some experiments have been made on the assumption that the same amount of protein will be precipitated by an increment of salt whether it be added, alone

or in multiples, to its own beginning concentration or to the beginning concentration of another salt. Preliminary data indicate that for mixtures of sulfates and of sulfates and chlorides the assumption is in general correct, or the deviations are not great. Mixtures of phosphates and sulfates have given unsatisfactory results so far, but not sufficient to be called negative.

TABLE III.

*Molecular Concentrations with Regard to PO_4 to Give Equal Na or K Concentrations for Sodium or Potassium Phosphate Equivalent to the Na or K Concentration at pH 7.0.**

	Protein fractions.			
	I	II	III	IV
Sodium phosphate mixture.				
Concentration of sodium, *mols*..	1.50	2.00	2.50	3.00
Mols of PO_4 ion. $\frac{NaH_2PO_4}{Na_2HPO_4}$ ratio.				
$\frac{8}{1}$	1.35	1.80	2.25	2.70
$\frac{1}{2}$	0.9	1.20	1.50	1.80
$\frac{1}{16}$	0.79	1.059	1.32	1.58
Potassium phosphate mixture.				
Concentration of potassium, *mols*......................	1.875	2.375	2.875	3.375
Mols of PO_4 ion. $\frac{KH_2PO_4}{K_2HPO_4}$ ratio.				
$\frac{8}{1}$	1.6875	2.1375	2.5875	3.375
$\frac{1}{2}$	1.125	1.425	1.725	2.025
$\frac{1}{16}$	0.966	1.223	1.481	1.739

* The ratios of monosodium (or potassium) phosphate to disodium (or potassium) phosphate used when in 0.15 molar solution have hydrion concentrations as follows: $\frac{8}{1}$ – pH 5.8; $\frac{1}{2}$ – pH 7.0; $\frac{1}{16}$ – pH 8.0.

DISCUSSION.

The data presented above bear upon an old problem. The first related study was made by Lewith (6), which was amplified and

discussed by Hofmeister (7). The subject has been discussed in detail by Spiro (8) and Chick and Martin (9), reviewed by Höber (10) and Robertson (11), and therefore another attempt will not be made here.

These experiments are comparable to those of Lewith and of Hofmeister in that the concentration of salt required for the completion of the precipitation of the first fraction should correspond very closely with the beginning of visible precipitation of protein which was used by these investigators as the index of the relative efficiency of different salts. Our index of equal efficiency is, however, more rigorous. The number of salts studied was smaller than might be desirable. With few exceptions no attempt has been made to study salts which are not capable of precipitating more than two of the globulin fractions. It is possible that with the extension of the suggestive results obtained with mixed salts we have a method of studying the relative precipitating efficiency of other less soluble salts. The data presented apply particularly to the comparison of the relative effects of the cations. Changes in hydrion concentration have not been studied except such as were present in the phosphate mixtures; the actual hydrion concentration in these cases is only known approximately. The precipitations took place in a range of hydrion concentrations in which considerable variations can occur without affecting the quantity of protein precipitated (9, 12, and our own experiments).

The results obtained have both a practical and a theoretical value. From a practical point of view, which is related entirely to the current acceptance of protein fractions obtained by salting-out, the number of salts which may be used for fractioning and studying the proteins of blood has been extended.

It is impossible to do more than to speculate on the theoretical significance of the data at this time, since an adequate conception of the nature of concentrated solutions is not available. Past evidence combined with that which is presented makes it apparent that the precipitation of protein fractions by means of salts under the conditions employed involves (*a*) both the cation and the anion of the salt, (*b*) for a given anion the cation is the determining factor in precipitation, (*c*) the valence of the cation is not a predominant

factor, and (*d*) for a given anion the order of magnitude of the concentration of the cation required is a multiple of that required for that of any other anion and is characteristic of the anion. The latter statement holds in general for the salts studied.

The relative efficiency of salts can be compared on two bases: (*a*) that used by Hofmeister in which we would use the completion of precipitation of the first protein fraction from plasma, and (*b*) the increment of salt or of cation required for each subsequent fraction. Our results agree in general with those of Lewith and of Hofmeister, with the exception of lithium sulfate and potassium phosphate which are required in higher concentrations than found by these authors. The determination of the concentration of salt required for the precipitation of the first fraction from blood plasma is open to some variation in case only that concentration is being determined. This is true because there is a zone, provided the concentration of the immediate protein fractions is not too large, of at least 1 per cent of salt in which there may be but slight change in the quantitative results. When the concentrations for the subsequent fractions are determined, however, this latitude is restricted since four points must be satisfied instead of one.

The quantity of chloride required for precipitation of the various fractions is of the order of five times the beginning concentration for the corresponding sulfate. This relation holds for sodium chloride, potassium chloride, and lithium chloride, and apparently for calcium chloride. If we assume that the quantity of magnesium chloride required to precipitate the first fraction is five times the concentration for magnesium sulfate then it is not possible to have sufficient magnesium chloride in solution to precipitate the first fraction; magnesium chloride does not precipitate the first fraction of blood. On the other hand, the addition of five times the increment of magnesium ion found for magnesium sulfate to a 0.75 molar solution of sodium sulfate will not give a precipitate with serum; a good precipitate is obtained with a solution containing 1.375 mols of sodium sulfate plus 1.875 mols of magnesium chloride.

When the various salts are compared on the basis of the increment between the various fractions after the precipitation of the first protein fraction of plasma, we find that for the sulfates, sodium and

potassium are equally effective, magnesium is next, and then lithium. The correspondence of the values for sodium and potassium may be of significance. Brönsted (13) has found the salting-out capacity of sodium and potassium ions to be very nearly equal, approximately 1 per cent higher for sodium than for potassium. Such a difference would not be readily demonstrable in our work; if it was present, we might still be able to obtain concordant results at all of the critical zones although a long series of experiments should show a deviation in one direction.

The relation between the increment of salt required for the succeeding fractions for the sulfates and chlorides does not seem to hold as regularly as for the beginning concentrations. With the exception of sodium and potassium chlorides, there are difficulties in working with the available chlorides as indicated in the experimental work. For sodium and potassium chloride the increment is five times the increment found for the sulfates. For lithium chloride the increment is two and one-half times the increment for lithium sulfate. With regard to the increment of the equivalent of base the values for the chlorides are two and one-half times the value for the sulfates in the case of sodium and potassium and one and one-quarter times the value for lithium. In the case of magnesium chloride the increment is apparently much larger than five times the increment for magnesium sulfate. The data cited with regard to the precipitation of the first protein fraction of blood serum by magnesium chloride and magnesium sulfate indicate that there is a much larger difference or that we were observing another phenomenon of mixed salts. On the other hand, there is no difficulty in mixing sodium sulfate and lithium or magnesium sulfate or in mixing sodium chloride and sodium or magnesium sulfate.

A relation has been sought by various investigators—see Washburn (14) and Höber (10) for a consideration of such work—between the hydration of the ions of a salt and its relative efficiency in salting-out, which has not always been found. From the data available on the hydration of the ions of salts and the results presented in this paper, it is apparent that so far as the relation existing in the solution, exclusive of the protein, is concerned salts with highly hydrated ions are not

so effective as those with less hydrated ions.[2] Thus the lithium ion is more highly hydrated than the sodium ion (15, 16) but sodium sulfate and sodium chloride are more effective in precipitating proteins than lithium sulfate and chloride, when compared on the basis of the beginning concentration. Potassium and sodium sulfates or chlorides are approximately equally effective, but the potassium ion is less hydrated than the sodium ion.

The recent work of Garrett and Lewis (17) indicates that the assumption that the water of hydration is always reserved for the exclusive use of the substance hydrated is open to question. These investigators found that the solvent power of the water of hydration of various substances may vary. They have suggested that "when both colliding individuals are hydrated, mutual solubility or penetration occurs. If only one individual is hydrated no generalization can as yet be made."

The data on the precipitation of proteins by mixtures of sodium or potassium phosphates indicate that for a given anion the cation is the effective agent. With essentially equal precipitation when the concentration of the cation was kept constant, the variation in the concentration of the PO_4 ion was sufficient to have precipitated practically all of the globulin present in blood serum, on the assumption that the cation and anion are equally effective. That the anion has an effect in the precipitation of protein is evident from data on the relative effectiveness of various anions when the same cation is present.

[2] It is possible that in the case of the high concentrations of the chlorides of lithium and calcium there is a relation between the relative ease with which these compounds produce insoluble protein precipitates and their pronounced tendency to form hydrates. Thus protein precipitated by lithium chloride is soluble in water, plus the salt present, for some time after precipitation but ultimately it becomes insoluble. The precipitate with lithium sulfate does not become insoluble within a reasonable length of time. With calcium chloride the action is much more rapid, if the concentration of calcium chloride is just sufficient to produce a good turbidity the precipitate can be redissolved by the addition of water for 2 or 3 hours afterward, but ultimately this is impossible. On the other hand, if a good flocculation is produced with calcium chloride (higher concentration), it is necessary immediately to add water to prevent the formation of an insoluble precipitate. The change appears to be progressive.

While data have many times been presented with regard to the specificity of action of ions in the salting-out of protein by the comparison of the effects of various cations having a common anion, so far as we can find no one has previously demonstrated that under certain conditions the action of one ion is relatively independent of the other by varying the quantity of one ion while the other ion is kept at the same concentration.

Sodium and potassium have been found to be equally effective as precipitants of protein with regard to the increment of base when combined with the sulfate or phosphate radicle. This is true in spite of the fact that the concentrations for beginning precipitation are different in the case of sodium and potassium phosphates, a difference which is too great to be accidental.

The evidence which Brönsted (13) has obtained from a study of the solubility of inorganic compounds in salt solutions has many points in common with the data presented in this paper on complex protein substances. The investigator finds:

"1. The activity coefficient of an ion may be determined by two factors, one of which is due to the salting-out effect of the salt solution serving as solvent and the other to electrical interaction between the said ion and the ions of the solvent.

2. Ions are uniformly influenced by ions of their own sign. Their activity coefficients depend, therefore, only upon the action of ions of opposite sign and the salting-out effect of the solvent.

3. The salting-out effect of a salt solution can be represented as a product of the salting-out effects of the separate ions."

Brönsted's data were obtained by comparing the solubility of various inorganic compounds in the presence of salt solutions of equal total ion concentration, whereas our data relate to the concentrations of salt which have equal salting-out ability. Under such conditions we have found that sodium and potassium salts of the same structure are approximately equally effective, and the ratios of the concentrations required as sulfate to those required as chloride are the same for these cations. The latter fact apparently holds for the beginning concentration for lithium chloride, but on the basis of the increment of salt lithium as a chloride is relatively twice as effective as when present as a sulfate. The relation between sodium and potassium

phosphates is not that which exists for the sulfates with regard to the beginning concentration, but it holds for the increment of salt. These facts agree in general with Brönsted's findings that the ratio of the salting-out capacities of two cations is the same for any anion and that a similar relation holds for two anions with a common cation.

An explanation will not be attempted at present of the salting-out of protein. The relatively limited knowledge with regard to the nature of concentrated solutions and the assumptions which must be made in order to account for certain effects in the salting-out of inorganic compounds do not admit of more than speculation with regard to the salting-out of proteins. We are inclined to the acceptance of the idea that the phenomenon is related to processes of solubility and salting-out which obtain with inorganic salts. This is essentially an acceptance of the fundamental conception first expressed by Spiro (8) and confirmed by Hardy (18), that salting-out could best be explained as a separation into phases. Spiro introduced the idea that there was a qualitative specific factor, solution intensity, which should be taken into consideration. Loeb (19) has recently presented evidence to show that the conditions which are responsible for the stability of proteins in solution and the precipitation of proteins by high concentrations of salts represent the forces of secondary valency, developed by Langmuir, responsible for the stability of crystalloids in general. On the basis of Loeb's distinction between colloidal properties and crystalloidal properties of protein solutions, our data are more in agreement with his evidence on the crystalloidal properties of proteins. McBain and Salmon (20) assume the possibility of soap existing in either the crystalloidal or the colloidal state.

The significance of the threshold concentration, *i.e.* the concentration of salt required to bring a protein solution to the point where the addition of an increment of salt will cause precipitation, and of the increment of salt must be left for further study. If the relation between the degree of hydration of the various proteins of blood and the quantity of salt required to precipitate a protein fraction holds, as suggested by Chick (21), then the quantity of water held by successive protein fractions is less for each fraction by an equal amount; her data do not show this relation. There is some question as

to the accuracy of the calculations based on her determinations, which does not, however, detract from the general value of the results.[8]

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